

Development of Novel Superparamagnetic Iron Oxide Based Theranostic Nanoparticles

by

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ABSTRACT

Superparamagnetic iron oxide nanoparticles (SPIONs) constitute popular nano-platforms in the continuously improving field of nanomedicine providing magnetic field dependent response. They are frequently investigated for opportunity for drug delivery, magnetic resonance imaging (MRI), hyperthermia and tissue engineering. Such popularity of SPIONs partially originates from their high biocompatibility. Indeed, SPIONs are the only clinically approved metal oxide nanoparticles. The ease of preparation, non-invasive diagnostic capability and suitability for further surface chemistries are another set of advantages in the medical field. All these properties make them potentially theranostic nanoparticles which can be adopted in various therapeutic and diagnostic modes of action in medicine but there seems to be more to explore in these novel compositions.

First part of this thesis summarizes the discovery of the intrinsically luminescent polyethyleneimine (PEI) coated SPIONs which provides gene-delivery combined with bimodal imaging modality. Such compositions have been studied frequently as transfection agents but their luminescence potential has never been considered or recognized before. Here, it will be shown that there is a tremendous and method dependent improvement in the poor blue luminescence of branched polyethyleneimine (bPEI) when coated on the surface of SPIONs. This novel finding is interesting especially when the quenching capability of iron oxide core in the visible range of the electromagnetic spectrum is taken into account. Carefully conducted spectroscopic and theoretical studies in this thesis tried to uncover the origin of such enhancement and provide an important contribution to the literature of luminescent polyamines. Ultimately, the amine oxidation, the restriction of bPEI upon its surface adsorption coupled with final acidification is the major combinatory factor behind

such a strong emission observed from bPEI-SPIONs. Besides, the transfection study of therapeutic oligonucleotide, poly I:C to the cervical cancer cell line HeLa demonstrated the potential theranostic utility of such nanoparticles. In short, this crucial finding, which is about the widely used bPEI coated SPIONs in the nanomedicine, both serves as a conceptual improvement in the literature of the fluorescent polyamines and enhances the scope of bPEI-SPIONs by showing their new diagnostic capability.

Second part of this thesis is devoted to an effort to develop a non-toxic, non-cationic, protein based transfection agent containing SPIONs as the carrier and signaling moiety. One of the most significant obstacles in the field of nanomedicine is the restriction of the gene carrier nanoparticles to the toxic poly-cationic compositions in order to improve their gene loading efficiency. This is a major drawback which particularly frustrates the notable biocompatibility of SPIONs. Therefore, safer and highly functional SPION based gene carrier designs need to be developed by excluding polycations to take advantage of the biocompatibility of iron oxide core to the full extend. With regards to this current problem, this thesis will demonstrate the development of a novel and multifunctional SPION based miRNA delivery agent utilizing a natural miRNA carrier protein *Argonaute 2* (AGO2) inspired by the natural miRNA carrying capability of AGO2 in the extracellular media. The ultimate nanoparticle was designed as AGO2 conjugated SPIONs tagged with an affinity tag (antiHer2 protein) and a far red emissive fluorescent tag (Cy5) to achieve a selective and image guided delivery of autophagy related miR376 therapeutics to the breast cancer models. At the second part of the thesis, the development of the small and colloiddally stable AGO2 and antiHer2-dye conjugated SPIONs were discussed with an optimized protocols. These nanoparticles show an excellent *in vitro* and *in vivo* biocompatibility and imaging guided *in vitro* and *in vivo* targeted delivery to the MDA-MB-453 and SKBR3 breast cancer cell lines. Besides, their capability for the delivery of miR376b mimics was confirmed by *in vitro*

analysis on MDA-MB-453 and SKBR3 which shows an increase in the level of native miR376b transcription. This work provides a first example to non-cationic, protein based SPION-transfection agent and miRNA-AGO2 complexation outside the biological environment.

Third part of this thesis work focuses on the delivery of SPION-based transfection agents to tumor tissue under the consideration of the intra-tumor heterogeneity. The molecular targeting of the therapeutics to the desired tissue is a new approach which reduces the side effects of therapeutics and improves their therapeutic efficiency. However, the tumors, especially those possessing the multiple molecular cues such as breast cancer, are heterogeneous and the cells even within the same tumor may show the overexpression of different receptors. In order to enhance the targeting efficiency of heterogeneous tumors, SPION immuno-conjugates which combine the antibodies sensing the overexpressed Her2 and EGFR signaling receptors on the surface of the breast cancer cells were developed in this thesis. Beside their specific molecular targeting capability, SPION immuno-conjugates take advantage of the superparamagnetic iron oxide core and can be directed under magnetic field which provides magnetic targeting as well. Bispecific SPION immuno-conjugates are anionic, colloiddally stable and small enough to circulate in the body. Targeting different breast tumor cell lines with such SPIONs are demonstrated in vitro. This approach contributes to a newly developing area of targeting nanoparticles to tumor heterogeneity.

In a conclusion, this thesis aimed to contribute to the literature of SPION based theranostics by providing new diagnostic capabilities, improving the biocompatibility and strengthening the molecular targeting issues.

ÖZET

Süperparamanyetik demir oksit nanoparçacıklar (SPIONs) ilaç taşınımı, manyetik rezonans görüntüleme (MRI), hipertermi ve doku mühendisliği gibi sürekli gelişen nano tıp alanlarında çok fazla kullanılan nano platformlardır. SPION ların bu kadar fazla ilgi görmesi yüksek biyo uyumlulukları ile ilişkilidir, öyle ki klinik onaylı tek metal oksit nano parçacık türüdür. Kolay sentezlenebilmeleri, yüksek ve noninvaziv görüntüleme imkanı vermeleri ve farklı yüzey kimyalarına açık olmaları da diğer avantajlarından bazılarıdır. Nano-teranostik alanına hatırı sayılır katkılarına karşın, SPION' ların potansiyeli, yeterince özgün kombinasyonlar kullanılarak değerlendirilmemektedir.

SPION ların görüntülenme kapasiteleri MRI ile kısıtlıdır. Fakat sonradan eklenen ve demir oksit çekirdeğinden ötürü zaman içinde ışıması kaybolan floroforlar ile çoklu görüntülenme imkanları yaratılabilmektedir. Buna karşın, luminesan bileşikler ile demir oksit çekirdeğinin etkileşimi, organik floroforun demir oksit üzerinde ışıma kaybına uğradığını gösteren birkaç çalışma dışında yeterince açığa çıkarılmamıştır. Özellikle, demir oksit- poliamin kombinasyonları literatürde gen aktarımında sıkça kullanılmasına karşın, demir oksit çekirdeğinin poliaminlerin zayıf ışımasına nasıl etki ettiği çalışılmamıştır. Bu noktada, dallı polyetilenimin (bPEI) kaplı demir oksit nano parçacıklar üzerine olan çalışmamız bPEI'e ait zayıf, mavi ışımanın demir oksit çekirdeği üzerine kaplandığında çok yüksek ölçekte ve metoda bağlı olarak arttığını ve uyarılma dalga boyuna bağlı ışıma gösterdiğini ortaya çıkarmıştır. Bu özgün bulgu, özellikle demir oksit çekirdeğinin elektromanyetik tayftaki görünür dalga boyunu soğurduğu bilgisi dikkate alındığında ilginçtir. Titizlikle sürdürülmüş spektroskopik ve teorik çalışmalar, bPEI'e ait zayıf ışımanın bu denli artmasının nedenlerini ortaya çıkararak poliamin literatürüne önemli katkıda bulunmuştur. Sonuç olarak, PEI'e ait

amin gruplarının yükseltgenmesi, nano parçacık yüzeyine tutunan bPEI' nin moleküler hareketliliğinin kısıtlanması asidifikasyon etkisi ile birleşerek bPEI/SPION a ait bu güçlü ışımanın arkadaşındaki temel etkeni oluşmuştur. Ayrıca, bu kendinden mavi ışığa özelliğine sahip SPION'ların teranostik potansiyeli de, PEI üzerindeki amin gruplarının yükseltgenmesinin bPEI/SPION un gen taşıma ve MRI sinyali üretme özelliklerini kayba uğramadığını temin etmek amacıyla test edilmiştir. Beklenildiği üzere, bahsedilen bPEI/SPION ların hücre içinde güçlü mavi ışığa verdikleri konsantrasyonda, kayda değer T2 MR sinyali de ürettikleri kaydedilmiştir. Buna ek olarak, söz konusu amin gruplarındaki kimyasal yükseltgenmenin PEI'nin gen taşıma işlevinin önünde engel teşkil etmediği, model oligonükleotit poly I:C nin servikal kanser hücre hattı HeLa ya başarılı bir şekilde aktarılmasıyla gösterilmiştir. Kısaca, nano tıpta yaygın olarak kullanılan bPEI kaplı SPION lar hakkındaki bu önemli bulgu, hem luminesan poliaminlere ait literatüre konseptsel gelişme sunmuş, hem de bPEI kaplı demir oksitlerin nanoparçacıkların yeni görüntüleme yeteneğini açığa çıkararak kapsamını genişletmiştir.

Nano tıpta karşılaşılan en önemli problemlerden birisi, gen taşıyıcı nano parçacıkların gen taşıma verimlerinin yüksek tutulması amacıyla, toksik poli katyonik bileşenler ile sınırlandırılmasıdır. Bu durum, SPION ların yüksek biyo-uyumluluk potansiyeline özellikle engel teşkil etmektedir. Bu nedenle, demir oksit çekirdeğinin gösterdiği biyo-uyumluluk özelliğinden bütünüyle yararlanmak için, polikasyonik yapıların bertaraf edilmesiyle daha güvenli ve yüksek fonksiyonellikle SPION bazlı gen taşıyıcı tasarımların geliştirilmesine ihtiyaç vardır. Gen aktarım çalışmalarında yer alan bu güncel problem ile ilgili olarak, özgün, çok fonksiyonlu, SPION bazlı mikro RNA (miRNA) ilaç taşıyıcı ajanlar daha güvenli gen aktarımı gerçekleştirmek amacıyla tarafımızdan ileri sürülmüştür. Bu motivasyonla, hücre dışı ortamda miRNA taşıdığı bulgusundan hareket ederek, doğal miRNA taşıyıcı protein *Argonaute 2* (AGO2) nun SPION yapılarıyla birleştirilmesi amaçlanmıştır. Bu özgün

tasarımların miRNA aktarma potansiyelleri, hedeflenebilir ve görüntülenebilir olması yaklaşımıyla meme kanserine karşı test edilmiştir. Bu amaçla, hedefleme etiketi (antiHer2 proteini) ve uzak kırmızı bölgede ışımaya gösteren floresan etiket (Cy5), AGO2 bağlanmış SPION lara eklenmiştir. Nihahi ürün, otofaji ile ilintili miR376b terapötiklerinin Her2 büyüme reseptörünü yoğun salgılayan meme kanseri hücrelerine seçici ve görüntüleme eşliğinde aktarılmasını sağlamak üzere tasarlanmıştır. SPION- protein kompleksi, içeriğindeki her bir yapı taşının işlevselliğini korumak üzere en etkin biyo-konjugasyon teknikleri kullanılarak elde edilmiştir. Geliştirilen SPION-protein birleşenleri Her2 reseptörünü yoğun olarak salgılayan MDA-MB-453 ve SKBR3 meme kanseri hücrelerinde mükemmel *in vitro* ve *in vivo* biyo-uyumluluk ve hedefleme özellikleri göstermiştir. Ayrıca, SPION-protein konjugatlarının miR376b mimiklerini aktarabilme özellikleri, MDA-MB-453 ve SKBR3 hücreleri ile yapılan ve yerel miR376b lerin seviyesindeki artış şeklinde tayin edilen *in vitro* testler ile doğrulanmıştır. Söz konusu SPION ların *in vivo* miRNA aktarma verimleri ise halen yürütülen meme kanseri tümör küçültme çalışmalarıyla değerlendirilmektedir. Bütün olarak ifade edildiğinde, geliştirilen anyonik, ultra küçük, koloidal olarak kararlı ve çok fonksiyonlu SPION-protein birleşenleri, AGO2-miRNA kompleksleşmesini ilk kez canlı dışı ortamda gerçekleştiren literatürde öncül formülasyonlardır. Bununla birlikte, söz konusu SPION-protein birleşenleri güvenli ve başarılı miRNA aktarım çalışmaları için güçlü adaylardır.

Moleküler hedefleme, yeni jenerasyon teröpatikler tarafından sunulan ve daha az doz ile daha yüksek teröpatik etki görülmesini sağlayan gelişmiş bir stratejidir. Ancak sözü edilen yetkinliğine rağmen, hedefleme yaklaşımı kanser hücrelerindeki her bir afinite birleşeni göz önüne alınarak geliştirilmelidir. Bu gelişmiş hedefleme uygulaması, özellikle meme kanseri gibi birden çok moleküler belirtisi olan hastalıklar için değerlidir. Meme kanseri, hücre yüzeyinde yoğun salgılanan birden çok tipte reseptörün varlığı sebebiyle önemli derecede

heterojen bir hastalıktır. Bu nedenle, tek tip hücre yüzeyi reseptörünü hedeflemek bazen tek bir tümörde dahi birden fazla hücre tipi bulunduran meme kanseri için çalışan bir yöntem değildir. Bu amaçla, meme kanseri hücreleri yüzeyinde yoğun salgılanan Her2 ve EGFR sinyal verici reseptörleri aynı anda tanıyan SPION-antikor birleşenleri tarafımızdan geliştirilmiştir. Çift dalga boyunda ışıma gösteren (yeşil ve uzak kırmızı) ve çift hedefli bu SPION- antikor birleşenleri, yine bio-konjugasyon teknikleri esas alınarak tasarlanmıştır. Moleküler hedefleme yeteneklerinin ötesinde, SPION-antikor birleşenleri süperparamanyetik demir oksit çekirdeğinin varlığının avantajı ile manyetik olarak da hedeflenebilmektedir. Çift hedefli SPION-antikor birleşenleri anyonik, koloidal olarak kararlı ve vücut içerisinde dolaşabilecek kadar küçük olarak elde edilmişlerdir. Söz konusu SPION-antikor birleşenlerinin hedefleme verimini göstermek için, halen sürdürülmekte olan ve farklı düzeyde Her2 ve EGFR salgılayan meme kanseri hücreleri üzerinde yapılan görüntüleme bazlı (FACS) hücre içi alımlı testleri tasarlanmıştır. *in vitro* testlerin tamamlaması ile, çiftli hedeflenebilen SPION ların moleküler hedefleme yaklaşımını daha spesifik ve daha akıllı hale getireceği öngörülmektedir.

Özetle, bu tez, eski SPION kompozisyonlarına yeni görüntüleme seçenekleri sunarak, miRNA taşıyıcı SPION ların biyo-uyumluluğunu önemli ölçüde yükselterek ve moleküler hedefleme stratejilerini güçlendirerek literatürde yer alan SPION bazlı teranostik çalışmalara katkı sağlamayı amaçlamaktadır.

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NOMENCLATURE

Fe ₂ O ₃	Maghemite
Fe ₃ O ₄	Magnetite
APTMS	Aminopropyltrimethoxysilane
EDC	Ethyl-3-(3-dimethylaminopropyl)carbodiimide
NHS	N-hydroxysulfosuccinimide
RES	Reticuloendothelial system
EPR	Enhanced permeability and retention
B	Magnetic induction
H	Magnetic Field
SMCC	Sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.
LC-SPDP	Sulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexonate
ICP-MS	Inductive coupled-mass spectroscopy
PEG	Polyethylene glycol
DLS	Dynamic Light Scattering
bPEI	Branched polyethyleneimine
ESR	Electron spin resonance

SPION	Superparamagnetic iron oxide nanoparticle
LA	Lauric Acid
NH ₄ OH	Ammonium hydroxide
IR	InfraRed
LE	Ligand Exchange
PET	Positron emission tomography
ΔG	Gibb's Free Energy
PAMAM	Polyamido amine
EWDE	Excitation wavelength dependent emission
MRI	Magnetic Resonance Imaging
TMX	Tamoxifen
miRNA	Micro RNA
PIC	Polyinosinic: polycytidylic acid
DNA	Deoxyribonucleic acids
T1	Longitudinal, spin – lattice relaxation
T2	Transverse, spin-spin relaxation
Γ_N	Neel relaxation time
Γ_B	Brownian relaxation time
k_b	Boltzman constant

γ	Surface Energy
$\Delta\mu$	Supersaturation
PDOT	Polymeric nanoparticles
EDTA	Ethylenediaminetetraaceticacid
PL	PhotoLuminescence
QY	Quantum Yield
MES	2-(N-morpholino)ethanesulfonic acid)
PBS	Phosphate buffer saline
MWCO	Molecular weight cut off
QD	Quantum Dot
QY	Quantum Yield
RT	Room Temperature
His	Histidine
PES	Polyethylene sulfonate
BSA	Bovine serum albuimin
TEM	Transmission Electron Microscopy
AGO2	Argonaute 2
PAA	Polyacrylic acid
UV	Ultra Violet

IVIS	<i>In vivo</i> imaging system
Vis	Visible
XPS	X-ray Photoelectron Spectroscopy
XRD	X-ray Diffraction
CES	Carboxyethylsilanetriol
DMSA	Dimercapto succinic acid
PDI	Polydispersity index
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
q-PCR	Quantitative polymerase chain reaction
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
VSM	Vibrated sample magnetometry
Her2	Human epidermal growth factor receptor 2
EGFR	Epidermal growth factor receptor
MI	Maleimide
TGA	Thermogravimetric analysis
μ	Magnetic dipole moment

CHAPTER I

1. INTRODUCTION

1.1. Magnetic Phenomena at the Macroscopic Level

Magnetism is the phenomenon associated with the charged particles moving through a space or the motion of electrons in an atomic orbital and it is mediated by the *magnetic field*. Magnetic field is an energy gradient which generates a force. This force can be 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 certain types of atoms. The most basic parameter in a magnetic system is the *magnetic dipole moment*. It can be simply described as a vector quantity measuring the torque generated by a magnetic field. Within the same idea, an orbiting electron also produces a magnetic moment. When an external magnetic field is applied, each moment experiences a torque tending to rotate itself through the direction of magnetic field. Magnetization capability of materials arises from these single orbiting electrons. [1, 2]

Behavior of magnetic materials in applied magnetic field is an important characteristic. This can be represented with the plot of *magnetic induction* $\mathbf{B}$ (flux density per square meter) for applied magnetic field $\mathbf{H}$ at various strengths. Alternatively, *magnetization* $\mathbf{M}$ (vector field representing density of induced magnetic dipole moments) against H can be also plotted with the given formula of $\mathbf{B}=\mu_0(\mathbf{H}+\mathbf{M})$ where μ_0 is the permeability of free space. The M versus H loop of magnetic materials provide important information about the magnetization properties of materials. These plots produce *hysteresis loops* which shows the magnetization capability of materials under applied magnetic field. (Figure 1.1) In a hysteresis loop, magnetization saturation is the upper limit of the magnetization that can be achieved below the Curie point.

Width of the loop is twice of the *coercivity* which is the reverse field applied to reduce the magnetic induction to zero. Another important parameter in the hysteresis loop is the *remanence*. In order to define what happens to the magnetic induction of a magnetic specimen after magnetic field is switched off, the term remanence is used. This remaining magnetic induction inside the material at the zero magnetic field is called as the remanent induction. The ratio of magnetization over the magnetic field is equal to the *magnetic susceptibility*. Magnetic susceptibility is an important parameter ranking the magnetization capability of materials. On the other hand, magnetization capability of materials has to be evaluated by taking some environmental considerations into account. Magnetization is a heavily temperature dependent property and oriented spins are able to get randomized when the necessary amount of energy is applied to the system. Therefore, a magnetically ordered specimen can become relaxed at elevated temperatures. This transformation temperature is called as the *Curie temperature*. [1, 2]

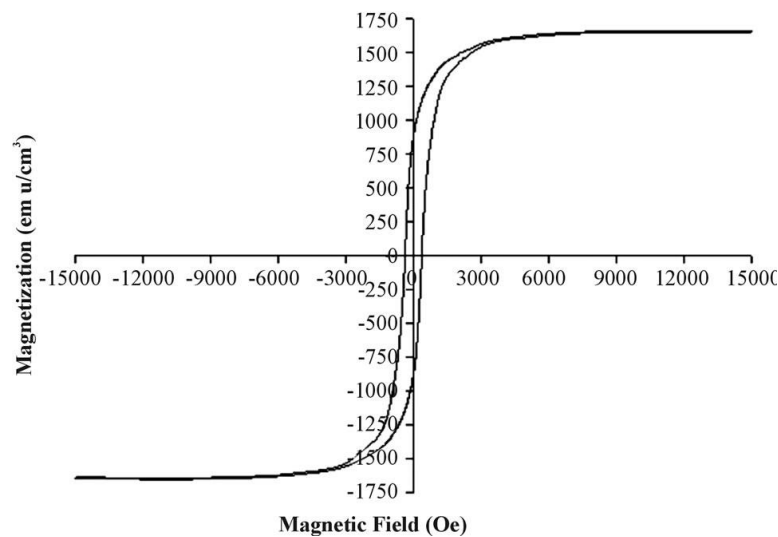


Figure 1. 1 Magnetic hysteresis loop of a typical iron film. [3]

1.1.1. Classes of magnetic materials

There are various crystalline and disordered magnetic materials exhibiting spontaneous long range magnetization arising from the localized magnetic moments below a critical temperature. Magnetic behavior of materials can be classified with the following phenomena : diamagnetism, paramagnetism, ferromagnetism, anti-ferromagnetism and ferrimagnetism. *Diamagnetism* is the fundamental property in all matters in nature and coming from the non-cooperative orbiting behavior of electrons under magnetic field, therefore, no net magnetic moment exists in diamagnetic materials when exposed to magnetic field. Like diamagnetism, *paramagnetism* is not a cooperative phenomenon of orbiting electrons either. Differently, materials exhibiting paramagnetism contain atoms with odd electron number and have a net magnetic dipole.[2, 4] However, magnetization arising from these individual magnetic moments disappears when magnetic field is removed. In addition, existing magnetic moments in paramagnets resist the randomizing effect of temperature, therefore, paramagnetic materials exhibit temperature sensitive magnetization. Transition metals with partially filled inner shells and their salts are paramagnetic compounds. Unlike paramagnetic materials, in a *ferromagnet*, electron spins are strongly interacted with each other and they are oriented totally in the same direction below a critical temperature. This gives a large spontaneous magnetization in ferromagnetic materials. If the system is completely isotropic, there exists a net magnetic moment in the arbitrary direction. However, above the Curie temperature, ferromagnetic materials act as a paramagnet. Iron, nickel, cobalt are typical ferromagnetic materials. *Antiferromagnetic* systems contain two or more equivalent sub-lattices which are interpenetrated. Below the transition temperature these sub-lattices are ferromagnetic and create collinear magnetic moments in the opposite directions. *Ferrimagnetic* materials contain several sub-lattices with a finite moment. Since the magnitudes of magnetic moments in these sub-lattices are not equal, there is not a complete cancellation of magnetization. [2, 4]

1.2. Magnetic Phenomena at the Microscopic Level

At the microscopic level, magnetization is a quantum mechanical phenomenon which depends on the electron-electron interaction in solids.[4] In bulk magnets, magnetization is not isotropic. Uniformity of spin alignments occurs only in the distinct small portions of the material. These small portions are called as *magnetic domains*. The magnetic domain concept is important in magnetism, because the size of each domain influences the magnetic properties of the material. Each domain nearly contains 10^{17} to 10^{21} atoms which are spontaneously magnetized and produce a magnetic moment. However, there are usually a large number of magnetic domains in a crystal lattice which either cancel each other or remained magnetized due to the large number of unpaired electrons .[1, 4] Ferromagnetic materials comprise a large number of magnetic domains within their lattice structures and are magnetized due to the large number of aligned spins when exposed to a magnetic field. An important characteristic of ferromagnets is their tendency to stay magnetized to some extent once the external field is overed due to the interaction of these domains. This generates the hysteresis in the B-H loop. Paramagnetic materials does magnetize in external magnetic field their magnetization is much weaker than ferromagnets. Due to the weaker interaction between the lower number of magnetic dipoles, paramagnetic materials become demagnetized when the magnetic field is removed. Therefore, paramagnets do not show a significant hysteresis in their B-H loop. [2] In brief, magnetic domains are significant parameters explaining the differences in magnetic properties of materials at the microscopic level.

1.2.1. Superparamagnetism : The Effect of Size on Ferromagnetism

There are two fundamental energies influencing the magnetization capability of a substance :

1) exchange energy arising from Pauli Exclusion Principle and producing ferromagnetic coupling 2) thermal energy producing the spin relaxation. When the amount of thermal energy, $k_b T$, is higher than the exchange energy, ferromagnetic alignment is destroyed.[5] The

dynamics between exchange and thermal energies are strongly dependent on the magnetic domains in a material. If the number of atoms decreases per domain, the interaction energy could reach the level of thermal energy at room temperature. This leads to spontaneous random orientation of electron spins, in other words magnetization disappears at zero field. *Herein, the critical factor is the number of atoms per domain and this could be achieved as the material size is reduced from bulk to nano.* Remanent magnetization in ferromagnets is cancelled at zero field when number of atoms per domain is significantly reduced inside the material. This means, material exhibits no hysteresis and acts as a paramagnetic material. Such a behavior is explained as *superparamagnetism* and could be observed in iron oxide at the sizes lower than 20 nm or nearly at 3 nm for pure iron. [6] Superparamagnets show high magnetization saturation with zero hysteresis, in other words, they incorporate the advantages of both ferromagnetism and paramagnetism in the same formulation. (Figure 1.2a-b) A ferromagnet and ferrimagnet can also act as paramagnet *above* the Curie temperature, but superparamagnetic behavior is observed *below* the Curie temperature. In a superparamagnetic material below the size of 20 nm, each nanoparticle act as a tiny magnet. Such particles do not possess any magnetization at zero field due to thermally induced spin relaxation. When their size overcomes the threshold value of 30 nm, nanoparticles act as a single domain ferrimagnets with some remanent magnetization. Above 80 nm, nanoparticles are divided into multi-magnetic domains and reach the highest coercivity. [7] Therefore, size of a superparamagnet has vital importance in its magnetic behavior.

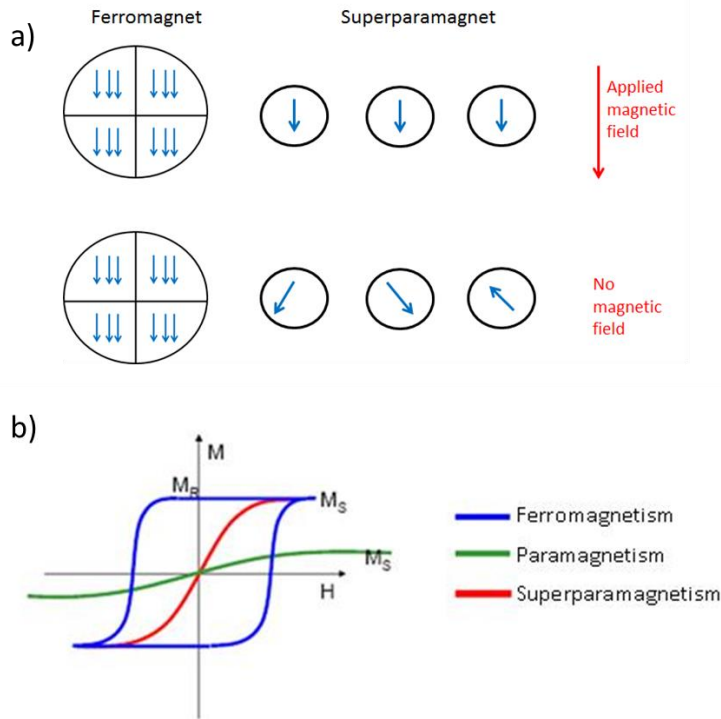


Figure 1. 2 a) Magnetic domains in ferromagnets and superparamagnets at applied and zero magnetic field. **b)** Representative magnetization curves for ferromagnetism, paramagnetism and superparamagnetism.

Superparamagnetic nanoparticle systems act as magnetic fluids. Each particle in a single domain generates local magnetic field and this leads a magnetic dipole-dipole interaction between particles. In this manner, nanoparticles in a defined volume are aligned through an external magnet and repulsive forces are present perpendicular to the external magnetic field. Under magnetic field, magnetic and Stoke`s forces act on a magnetic body. While magnetic force causes the acceleration of the particles, Stoke`s force hinders the movement by viscosity. Magnetic force **(1)** and Stoke`s force **(2)** can be expressed by the following equations. [6]

$$F_m = \frac{4}{3} \pi r_m \nabla (\vec{M}(z) \vec{B}(z)) \quad (1)$$

Where r_m is the radius of the magnetic part of the particles, M is the magnetization of the particle and B is the magnetic flux.

$$F_s = 6\pi\eta r_h v \quad (2)$$

Where η is the viscosity of the liquid, r_h is the hydrodynamic radius of the particle, v is the velocity of the particle.

From the given equations, it should be noted that hydrodynamic radius of the particle could be larger than the magnetic radius. This is especially valid for the particles coated with organic ligands. Coating the crystalline magnetic nanoparticles with organic species has vital importance for the colloidal stability of the nanoparticles which has a critical importance in the application of such materials in the physiological fluids. In order to obtain colloidal stability and overcome the attractive forces including magnetic dipole-dipole and van der Waals interactions, repulsive forces need to be generated in the carrier liquid. Repulsive forces depend strongly on the ionic strength and pH of the media and the type of the coating material. [6] Therefore various approaches have been developed to produce colloidally stable superparamagnetic nanoparticles.

1.3. Superparamagnetic Iron Oxide Nanoparticles (SPIONs)

SPIONs are nano sized crystalline iron oxides in the range of 10-100 nm. [8, 9] Because of their small size, these magnetic colloids act as a single domain materials [8] Two important relaxation mechanism have to be considered for colloidal SPIONs: Brownian rotation with a relaxation time τ_B (3) where v is the volume of the particle, η_0 is the viscosity of the liquid, k is the Boltzman constant and T is the temperature. *Brownian relaxation* is, therefore, temperature and viscosity dependent which determines the basic rotational mobility of colloidal particles. [10] Second relaxation is *Neel relaxation* which is caused by the random

deorientation of spins under the influence of temperature.[11] Neel relaxation time Γ_N (4) where K is the anisotropy constant, f_0 is the Larmor frequency, about 10^9 s^{-1} , is heavily volume dependent and dominant when the size of nanoparticles is smaller than nearly 20 nm.

$$\Gamma_B = \frac{3v\eta_o}{kT} \quad (3)$$

$$\Gamma_N = f_0^{-1} e^{\frac{Kv}{kT}} \quad (4)$$

As it is denoted by the equations (3) and (4), behavior of SPIONs are dictated by the thermodynamics, so the magnetically fluidic system adapts itself to minimize its free energy.

SPIONs have two main crystal structures : magnetite and maghemite.[12] Magnetite belongs to spinel form of minerals and it takes cubic inverse spinel structure, namely the oxygen ions are placed on a closed packed cubic lattice with the tetrahedrally and octahedrally located iron ions on the intersects. [13] The chemical formula of magnetite is $\text{FeO} \cdot \text{Fe}_2\text{O}_3$ with high intrinsic magnetic moment per unit cell. In SPIONs, magnetic moment arise from the unpaired 3d electrons in Fe^{3+} and Fe^{2+} cations. Electron spin coupling between Fe^{3+} and Fe^{2+} takes place on the octahedral sites and antiparallel couplings exist on tetrahedral sites. Exchange interactions between these two sites across the oxygen anions are called as *superexchange* interactions and they are responsible for the magnetic behavior of SPIONs. Superexchange interactions might be destroyed due to oxygen vacancies, cationic vacancies, edge roughness or disruption of local symmetry with a magnetically dead layer. These abnormalities significantly affect the magnetic properties of SPIONs and they are sensitive to size, shape, crystallinity and surface coatings. [14]

1.3.1. The Relationship between Physical and Magnetic Properties of SPIONs

The reduction in the size decreases magnetic moments in SPIONs.[15, 16] In addition, decrease in size improves the surface to volume ratio and promotes the “spin canting effect”, namely distortion of spins on the surface electrons.[17] SPIONs around 5 nm diameter exhibit surface canting effect at one atomic layer. The thickness of spin canting layer of 11 nm sized SPIONs rises up to two atomic layers. However, surface canting layer fell significantly for large nanoparticles (>11 nm).[17] In addition to spin canting effect, crystal defects decrease the magnetization of SPIONs at reduced sizes.[18] Shape is another significant factor controlling the magnetic properties of SPIONs. Generally, spherical SPIONs show much reduced spin canting effect compared to cubical or ellipsoidal SPIONs and accordingly exhibit enhanced magnetization values. [19, 20] Differently, it was shown that mixed shaped SPIONs show magnetization values near their bulk counterparts. [21]

Organic/inorganic capping agents form a protective layer around SPIONs via their end groups through electrostatic interactions or covalent bonding. The thickness of protective layer changes from 1-5 nm to 10-100 nm depending on the monomeric or polymeric surfactants respectively.[22] The capping agents comprises of different end groups such as $-\text{OH}$, $-\text{COOH}$, $-\text{NH}_2$, $-\text{PO}(\text{OH})_2$, $-(\text{SO})_2-\text{OH}$. Magnetic properties of SPIONs differ with the various type of capping ligands. For example, Dabandi *et al* showed that $-\text{OH}$ functional polyoxyethylene (5) nonylphenylether has negligible effects on the magnetic properties of SPIONs because capping agent does not distort surface structure of magnetic nanoparticles.[23] However, contrarily, spin canting measurements showed that polyoxyethylene (5) nonylphenylether reduces the average surface spin canting arising from the affinity of $-\text{OH}$ groups towards the surface atoms on the nanoparticles. Another significant observation was reported by Roca *et al* showing the increase in the surface canting effect on SPIONs when interacted with the oleic acid.[24] However, Guardia *et al* observed that surface spin disorder on SPIONs was

diminished when capped with oleic acid and high quality magnetic nanoparticles could be generated.[21] Mohapatra *et al* showed that surfactants with phosphonic acid groups have enhanced affinity to SPIONs due to the extra oxygen in the ligand.[25] Indeed, coating of SPIONs can be done during the synthesis or afterwards. Initial coating materials may be replaced later with other ligands more suitable for a specific application. Usually, direct coating provides better magnetic properties. This might be attributed to the reduced spin canting effect and binding affinity. [26] However, if this ligand exchange is performed with a more strongly bound ligand, properties may be improved as in the case of chatecoles displacing carboxylic acid containing ligands . It is suggested that this provides a partial realignment of surface spins which improves the magnetic moment of nanoparticles. (Figure 1.3) [22]

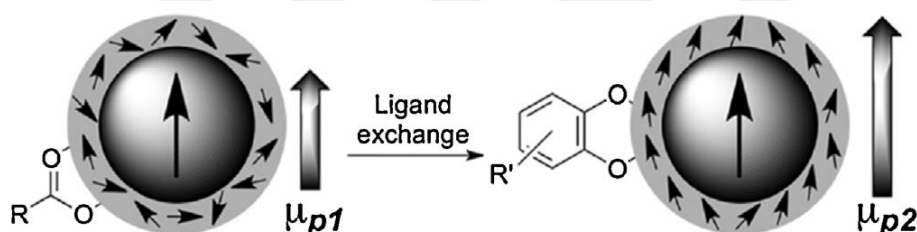


Figure 1. 3 Representative image showing the influence of capping agents on the overall magnetic moment of SPIONs. ($\mu_{p1} < \mu_{p2}$) [22]

In brief, the size, shape, crystallinity and surface coating are significant parameters determining the magnetic capability of SPIONs. These parameters are flexible and could be adjusted through the different synthetic approaches. Magnetization values of several SPIONs with different size, shape and surface coatings are summarized in the Table 1.1.

Table 1. 1 Size, shape, surface coatings and magnetization values of various SPIONs.

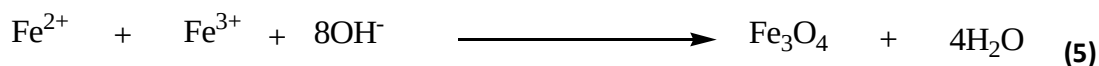
Size (nm)	Shape	Surface Coating	Magnetization (emu/g)	Reference
14±4	Spherical	Uncoated	74.9	[27]
18	Spherical	Oleic Acid	67	[28]
24	Mixed Shaped	Uncoated	89	[29]
21	Spherical	Hexamine	59	[29]
12.4	Mixed Shaped	Galactose	59	[30]

1.3.2. Synthesis Methods of SPIONs

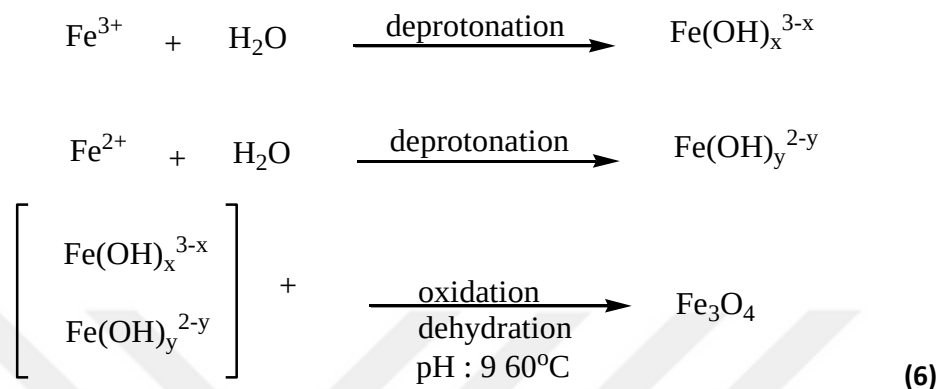
In the past decade, synthetic methods for SPIONs have been improved significantly in an effort to develop high quality particles for various technical applications such as magnetic storage media [31], bio-sensing [32], medical [33], bio-imaging [34] applications. Among them, physical methods such as gas phase deposition and electron beam lithography suffer from poor control on the nanoparticle size. [35, 36] Therefore, wet chemical methods are widely used to obtain SPIONs in more simple routes with more control on properties.[37, 38] Wet chemical techniques could be categorized as coprecipitation, thermal decomposition, hydrothermal, microemulsion and sonochemical methods.

Coprecipitation Method

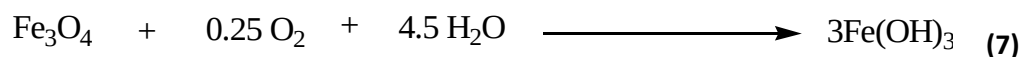
Since it provides a simple and an efficient synthetic method, coprecipitation is the widely used technique among the wet chemical routes for the synthesis of SPIONs. Magnetite and maghemite could be synthesized through the coprecipitation of ferric (Fe^{3+}) and ferrous (Fe^{2+}) salts under alkaline conditions and at elevated temperature. [39] The overall chemical reaction of coprecipitation process can be written as in equation 5. [40, 41]



Co-precipitation reaction begins with the hydrolysis of the iron salts. Detailed reaction mechanism is given in the equation 6:



Massart reported that the most efficient coprecipitation can be achieved at the pH range between 9 and 14 and with the molar ratio of $[\text{Fe}^{3+}] : [\text{Fe}^{2+}] = 2:1$ under inert atmosphere. [42] Otherwise, magnetite could be oxidized through the following equation 7.



The parameters of coprecipitation reaction were carefully studied to analyze the effect of base, pH, added cations and $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio on the diameter and polydispersity of the particles by Massart. [43, 44] When given parameters are changed, size distribution of nanoparticles from 4 to 16 nm was obtained. Jolivet *et al* observed formation of goethite with lower $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratios. At $\text{Fe}^{2+}/\text{Fe}^{3+}$ mole ratio of 0.3, two distinct phases were formed: 4 nm sized nanoparticles with low Fe^{2+} content and nonstoichiometric magnetite. In case of $\text{Fe}^{2+}/\text{Fe}^{3+}$ mole ratio of 0.5, homogenous magnetite nanoparticles were obtained. Babes *et al* also showed that increase in the $\text{Fe}^{2+}/\text{Fe}^{3+}$ ratio, leads into formation of larger nanoparticles.[45] Other important parameters are reported as the acidity and the ionic strength of the precipitation medium. [46, 42]

Thermodynamical approach to coprecipitation reaction : nucleation and growth mechanisms during the crystallization

Coprecipitation is a crystallization process of magnetites from the iron salts.[47] During the crystallization from the solution, nucleation and growth mechanisms take place for the formation of nanoparticles. [48, 49] Nucleation is a series of atomic processes by which atoms of reactant phase transform into a product phase in the form of clusters. The driving force of nucleation is the *supersaturation*. Supersaturation ($\Delta\mu$) depends on the chemical potential difference between the solution and bulk ($\Delta\mu = \mu_s - \mu_c$). If $\Delta\mu > 0$, system is supersaturated and nucleation/growth processes are favorable. When $\Delta\mu < 0$, the dissolution process is favorable. [49] According to the thermodynamics, the work which is necessary to form nuclei is the difference between the free energies of the initial and final states plus a term corresponding to the interface between nucleus and the solution. For a spherical particle, total free energy ΔG can be defined as in equation 8 where r is the radius of the particle, γ is the surface energy, ΔG_v is the free energy of the bulk crystal which is temperature dependent. [50]

$$\Delta G = 4\pi r^2 \gamma + \frac{4}{3}\pi r^3 \Delta G_v \quad (8)$$

Surface free energy is always positive and crystal free energy is always negative, so there is an optimum free energy favoring nucleation which depends on the particle radius. This radius is called as the *critical radius* and corresponds to the minimum size at which particle can survive in the solution without dissolution. [50, 49] Crystal growth is a series of mechanisms by which atoms are incorporated onto the surface of the nuclei and increase the size. Growth mechanism is controlled by two processes : surface reaction and monomer's diffusion to the surface. Crystal growth theories basically concentrate on the crystal surface structure. [51]

Although the classical nucleation and growth theories explain the energetics between these phenomena, they are not able to answer the differences in the sizes of particles during the growth. This situation has been tried to be explained with various theories. LaMer provided the most widely accepted theory about the nucleation and growth. [52, 53] LaMer mechanism is divided into three parts. (I) A rapid increase in the concentration of monomers in the solution which leads into (II) a burst nucleation to decrease the concentration of monomers significantly. After this point nucleation does not exist due to very low concentration of monomers. (III) Subsequently, diffusion controlled growth of nuclei starts through the solution. (Figure 1.4) According to the LaMer diagram, necessity to obtain monodisperse crystals is to separate the nucleation and the growth processes in time. Minimizing the nucleation time, namely processing a burst nucleation provides a large number of nuclei at nearly same size. This ensures that the entire nucleation takes places in that short time frame followed by much slower growth process, which separates nucleation and growth temporally.

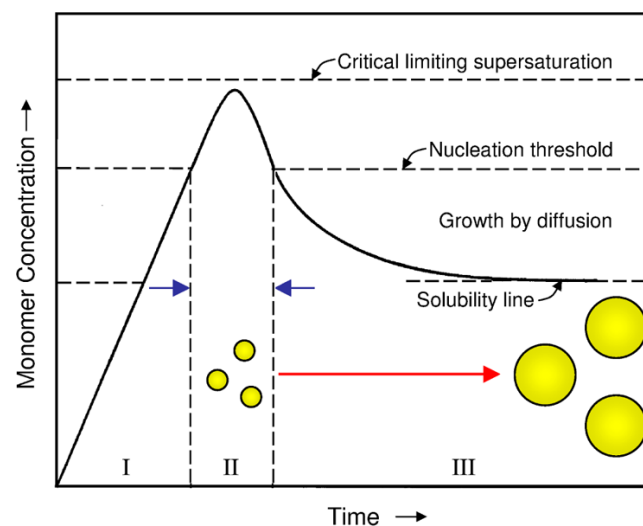


Figure 1. 4 Schematic representation of three stages in LaMer mechanism during the nucleation and growth. Zone I : Increase in the concentration of the monomers in the solution, zone II : nucleation, zone III : growth.[54]

Another important theory for growth is known as the *Ostwald Ripening* which was defined in 1900. [55] It explains the growth by the change in the solubility of nanoparticles. In general, surface to volume ratio of smaller particles is relatively high which leads a higher surface energy. Higher surface energy is not thermodynamically favored. Therefore, larger particles grow at the expense of smaller particles to reduce the surface energy in the solution which is not in the thermodynamic equilibrium. Diffusion controlled Ostwald Ripening is a predominant growth mechanism. Diffusion process is controlled by surface energy. The chemical potential at the solid/liquid interface is much higher for smaller particles which induces a concentration gradient from smaller to larger particles and allow larger particles to grow more efficiently than the smaller particles. (Figure 1.5)

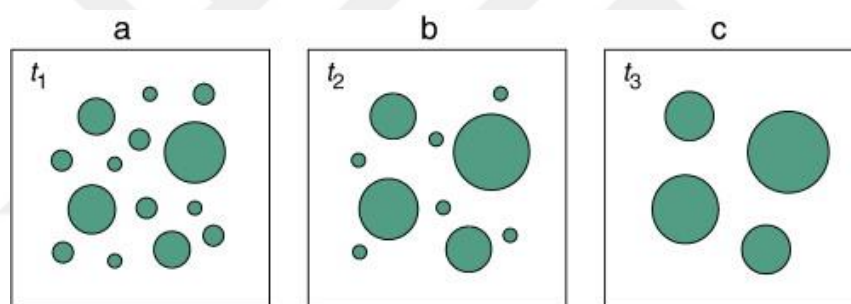


Figure 1. 5 Schematic illustration of Ostwald Ripening showing an increase in the particle mean size without changing the volume fraction of the coercive phase in time ($t_1 < t_2 < t_3$). [56]

Theoretical approaches to nucleation and growth mechanism have been tried to be supported by experimental evidence. Baumgartner *et al*, resolved the formation mechanism of magnetite by cryogenic transmission electron microscopy. [57] They observed crystallization pathway involving occurrence of nanometer sized primary particles that fused into large crystalline particles. The formation of primary particles takes place within the 2 minutes after the addition of precursors and are monodisperse 2 nm aggregates. In 6 minutes, aggregates become denser and form spheroidal particles in 10-15 nm size range.

The main advantage of the coprecipitation method is its applicability to large scale synthesis. However, it suffers from poor size and crystallinity control of the nanoparticles. Size distribution of nanoparticles is limited by the kinetically controlled growth mechanism.[58] Microemulsion methods wherein the growth is restricted within the water/oil droplets were reported to provide a tighter control on growth of nanoparticles.[59] However, its applicability to large scale synthesis of highly crystalline iron oxide nanoparticles is limited. [14]

Magnetic nanoparticles prepared under different experimental conditions display diverse magnetic properties. This is attributed to the change in structural disorder, existence of magnetically dead layer and antiphase boundaries. [60-62] Generally, saturation magnetization values of magnetic nanoparticles (30-50 emu/g) are lower than their bulk counterparts (90 emu/g). [63] Some reports showed that, such reduction in the magnetization could arise from the noncollinear spins at the surface defects since high surface curvature encourages the disordered crystal growth.[64] However, size, shape and size distribution of nanoparticles can be controlled by changing various parameters such as pH, ionic strength, temperature, injection rate, chemical structure and the stoichiometric ratio of the iron salts.[6, 14, 16, 20]

Thermal decomposition method

Thermal decomposition method provides highly crystalline and monodisperse iron oxide nanoparticles compared to coprecipitation method. In this method, SPIONs are synthesized in the presence of surfactants in higher boiling organic solvents. [65] usually the precursor is injected into the hot solution providing fast nucleation. However, this method produces hydrophobic nanoparticles since the surfactant is adsorbed on the crystal. Such SPIONs are not suitable for biomedical applications. Therefore, they need to be transferred to aqueous

phase either by the exchange of the surfactant with water soluble, biocompatible molecule or via formation of an interdigitating secondary layer on top of the initial surfactant coating. [66]

Hydrothermal method

This method is similar to coprecipitation in aqueous solution but performed at high temperatures and under pressure. SPIONs in various shape and size can be obtained through the hydrothermal synthesis. Crystallinity of the nanoparticles is poor compared to thermal decomposition method. [67]

Sonochemical method

Ultrasound can be used to synthesize SPIONs in aqueous media by the cavitation of the bubbles. Depending on the refluxing time, irradiation time and power the size and shape of the nanoparticles can be modified. [68]

Microwave assisted synthesis

SPIONs can be synthesized with the microwave energy in a very short time in a less energy demanding way. Uniformity of applied energy aids formation of monodisperse and highly crystalline nanoparticles. [69] SPIONs generated with microwave assisted synthesis show low surface reactivity which enhances their stability. [70]

1.4. Biomedical Applications of SPIONs

Superparamagnetism is a notable magnetic property especially in biomedicine because superparamagnets do not possess a remanent magnetization after removing the field. This provides a controllable movement in the blood under magnetic field when compared to the ferromagnets.[71] SPIONs with suitable surface characteristics can find various applications in biomedicine such as MRI (magnetic resonance imaging) contrast improvement, tissue

repair, drug delivery, gene delivery, hyperthermia and cell separation. [72] Other attractive properties of SPIONs in the biotechnology include high saturation magnetization [72], monodisperse hydrodynamic sizes smaller than 100 nm [73], functional surface coatings [33], biocompatibility [66] and biodegradability. Therefore, several factors including size, charge and surface chemistry has to be taken into consideration during the engineering of SPIONs when they are issued for biomedical purposes.

Nano-sized particles (< 100 nm) are desired to gain superparamagnetic nature and improve their bioavailability in vivo. Since magnetic dipole-dipole interactions are significantly reduced with the radius of the particles, iron oxide nanoparticles smaller than 30 nm are substantially superparamagnetic having much less exchange energy between the electron spins. [71] Another advantage of the nano-size effect is the higher surface reactivity arising from higher surface to volume ratio of the nanoparticles. This provides lower sedimentation in the blood stream, higher tissue penetration and escape from the reticuloendothelial system (RES). [74] Before clearance by RES organs such as macrophages, nanoparticles are surrounded by the plasma proteins in the blood and. Opsonized nanoparticles are rapidly cleared from the vessels. This process is named as *opsonization*. So, adsorption of the plasma proteins on the nanoparticles determines their biological fate. [75] Since, nanoparticles should remain in the blood stream before they arrive to target tissue, opsonization has to be prevented or reduced to provide a long blood circulation time. Size of the nanoparticles is one of the most significant pharmacokinetic parameter. Smaller particles usually have longer blood circulation time, because density of adsorbed plasma proteins on smaller nanoparticles is lower than on larger particles. For instance, it has been reported that only 6% of plasma proteins are adsorbed onto 80 nm sized nanoparticles while 34% of plasma proteins are adsorbed on 240 nm sized nanoparticles.[76] As a consequence, systemic clearance of smaller nanoparticles is delayed as compared to larger nanoparticles. Blood circulation half-life of

various iron oxide nanoparticles in clinics change from 30 mins to 30 h. As an example, Ferumoxide (80-150 nm) is cleared from the body in 30 mins, while Ferumoxtran-10 (20-40 nm) has much higher plasma half-life around 30 h. [77, 78] Here, it is important to note that these sizes are indeed the hydrodynamic size of the crystalline iron oxide crystals. This hydrodynamic size of nanoparticles determines their bio-distribution profiles, as well. Nanoparticles larger than 200 nm undergo a spleen filtration, nanoparticles smaller than 100-50 nm are generally cleared by hepatic parenchyma and nanoparticles smaller than 6 nm experience renal clearance. Much larger nanoparticles ($> 4 \mu\text{m}$) are internalized by lungs and may cause embolism. [79, 80] Size of nanoparticles is also crucial for the passive targeting of the nanoparticles through the enhanced permeability and retention (EPR) effect. EPR is a significant way to internalize the 100-150 nm therapeutic particles inside the solid tumor tissues exhibiting permeable vascularity due to the aberrant vascular architecture in tumors. [81] Overall, size of SPIONs have to be designed carefully for the specific purpose. In general, it is desired to tune the size big enough to prevent quick renal clearance and small enough to prevent quick clearance by RES, hence provide enough blood circulation to leak into tumor tissues by EPR.

Surface coating of SPIONs influence their blood circulation time and clearance, as well. In general, hydrophobic and charged particles are cleared more rapidly compared to hydrophilic and neutral particles. Opsonized particles are usually picked up by macrophages and accumulated in the RES organs such as liver and spleen. Antifouling shells can be introduced to generate a shielding effect. Polyethylene glycol (PEG) is the most representative polymer used to prevent the RES clearance of intravenously introduced nanoparticles. Extended PEG chains around the surface prolong the blood circulation time of nanoparticles by creating steric hindrance against the opsonins. [82]

1.4.1. Toxicity and Biocompatibility of SPIONs

Biocompatibility is one of the most significant criteria for the biomedical applications of SPIONs. Various magnetic materials such as cobalt, nickel, gadolinium are highly toxic and susceptible to the oxidation. Magnetite and maghemite are the only accepted metal oxides in clinical applications due to their low toxicities.[83] However, they also have potential risks related to immune response and cellular toxicity. Toxicity of iron oxide nanoparticles can be related to numerous factors such as dose, size, surface chemistry, chemical composition, route of administration, biodegradability, pharmacokinetics and biodistribution. Surface coatings significantly affect the cellular toxicity, and it has been shown that more biocompatible coatings such as proteins decrease cellular toxicity significantly [84]. Size of the nanoparticles has also critical impact especially on *in vitro* cellular toxicity. As an example, it was reported that 30 nm iron oxide nanoparticles are remarkably toxic compared to 0.5 μm nanoparticles.[85] This difference is coming from the change in the surface to volume ratio of nanoparticles. Since 30 nm nanoparticles have much higher surface to volume ratio, their interaction with the cellular compartments are much more facilitated and cause more rapid cellular damage. Surface charge dependent cytotoxicity of iron oxide nanoparticles arises from the higher attraction between the nanoparticles and the plasma membrane of the cells. In general, positively charged nanoparticles strongly interact with the blood components and undergo non-specific binding which causes severe toxicological effects such as cell lysis [86]. Therefore, positively charged particles are much toxic compared to neutral or anionic particles. Cellular toxicity of iron oxide nanoparticles is also directly related with the release of metal ions after their corrosions. (Figure 1.6) After endosomal degradation of iron oxide nanoparticles, free iron metals participate in the iron metabolism. These free irons are stocked in the iron stores of the body then found as hemoglobin or bound to transferrin. This is the origin of SPION biocompatibility. However, higher doses of iron metals in the body (higher

than nearly 0.1 mg/mL) may exceed the iron binding capacity of transferrin. Under these circumstances, SPIONs may evoke cellular responses such as DNA damage, oxidative stress, expression of various genes involving cell cycle regulation, iron homeostasis, pancreatic functioning or reorganization of actin cytoskeleton in in the long term. [87] Toxicity of iron oxide nanoparticles are more pronounced in the *in vitro* studies rather than the *in vivo*. Cells are directly and continuously exposed to the nanoparticles *in vitro*, on the other hand, nanoparticles slowly act on cells and continuously eliminated by the body *in vivo*. Therefore, toxicological effects of nanoparticles can be significantly differ between *in vitro* and *in vivo* studies. However, *in vitro* toxicity studies are the first step of biocompatibility evaluation which has to be followed by proper *in vivo* evaluations. Dose and route of administration are key factors in the *in vivo* evaluation of iron oxide nanoparticles. For example, intravenous injection of oleic acid stabilized SPIONs into rat at a dose of 10 mg Fe / kg is report as safe. Intraperitoneal injection of 50 nm SiO₂ coated magnetic nanoparticles did not elicit severe toxicity at doses between 25-100 mg Fe/kg. Subcutaneous injection of 15 nm dextran coated iron oxide nanoparticles are reported as safe at the dose of 3150 mg/ kg.

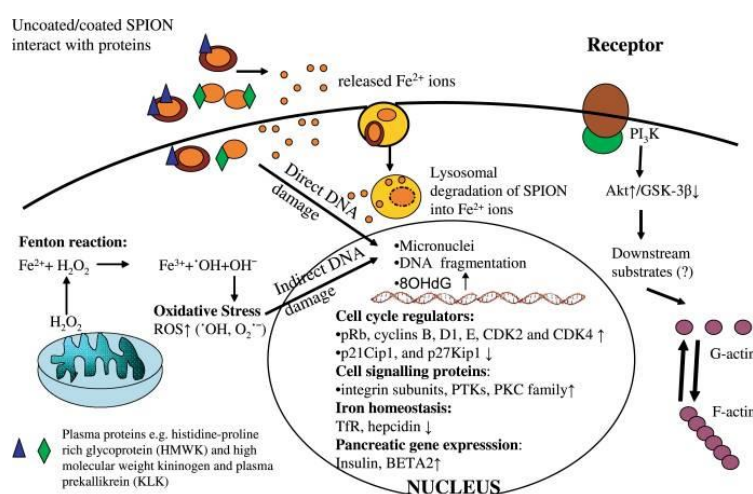


Figure 1. 6 Schematic representation of SPION induced cellular toxicity.[87]

As a consequence, preclinical and clinical assessments show that iron oxide nanoparticles can be considered as biocompatible. However, as it denoted their safety is directly related with their size, dose, and route of administration. Therefore, toxicity of iron oxide nanoparticles have to be evaluated case by case for the considered medical applications. Details of some medical applications are given in the following sections.

1.4.2. SPIONs in magnetic resonance imaging (MRI)

MRI is a non-invasive soft tissue imaging technique based on monitoring water content in the body. MR signals arise from the change in the alignment of hydrogen nuclei of water molecules under external magnetic field. When exposed to radio frequency nuclei of the waters are excited. Here, the frequency of the radio waves must be in resonance with the frequency of the hydrogen nuclei. Upon their return to the original positions, aligned nuclei of hydrogens release energy as radio frequency and this can be detected by MRI device. The realignment time of these hydrogen nuclei are different in various tissues and defined as relaxation times. T_1 (longitudinal, spin – lattice relaxation) and T_2 (transverse, spin-spin relaxation) are relaxation times needed for reorientation of the aligned vectors into their equilibrium positions and produce MR signals. However, short T_1 and T_2 are needed to generate contrast in MRI. With this aim, magnetic materials such as Gd-complexes or SPIONs are used to generate large microscopic magnetic field gradients which result in substantial diphasing and shortening of T_1 and T_2 relaxation times. These magnetic materials are called as *contrast agents*. Contrast agents are divided into two categories : T_1 agents which generally increases spin-lattice relaxation rates ($1/T_1$) of water protons more than spin-spin relaxation rates. This shortens the T_1 and increases the signal intensity providing a bright contrast. T_2 agents increase the spin-spin relaxations ($1/T_2$) and decrease the signal intensity. Therefore, they generate negative contrast, namely dark contrast. The type of a contrast agent (T_1 or T_2) depends on the type of the contrast agent and the aggregation as well as the imaging

sequence, field strength, correlation time and how the contrast agent is placed inside the tissue. The ability of a contrast agent reducing T_1 and T_2 times are referred as R_1 ($1/T_1$) and R_2 ($1/T_2$) relaxivity values. [88]

MRI contrast agents are described as paramagnetic and superparamagnetic compounds. Paramagnetic metal chelates and iron oxide nanoparticles are two significant classes generating MRI contrast in biological media. [89, 90] SPIONs have large relaxivity values compared to paramagnetic agents because they have larger number of aligned electron spins compared to a single paramagnetic agent. Relaxation mechanism of SPIONs is generally explained by susceptibility induced relaxation: when water molecules feel magnetic gradient around the nanoparticles, the protons are relaxed by outer sphere mechanism. Effective spin dephasing and transverse relaxation decrease the T_2 signal intensity and create a negative contrast. Due to their large size and magnetic moments, SPIONs are developed as negative (T_2) contrast agents. R_2/R_1 values determines the effectiveness of a contrast agent as dark or bright agent and this ratio increases with the size (aggregation) of SPIONs. Hence, most SPIONs are negative contrast agents. SPIONs are utilized for the imaging of various tissues such as liver, spleen and lymph nodes. [91-94] Additionally, SPIONs are also accompanied by perfusion imaging of the brain, myocardium, kidney or they can be even used in MR angiography to monitor tumor vascularization.[95] For angiography, SPIONs have to stay in the blood circulation much longer which can be achieved by reducing the size. Indeed, SPIONs can be also designed as T_1 contrast agents by reducing their size to around 10 nm. [96] There are various commercial iron oxide nanoparticles used as T_2 contrast agents for MRI : Feridex[®] (Berlex, USA), Endorem[®] (Guerbet, France), Resovist[®] (Shering AG, Germany). Some of the currently used SPIONs for MRI are summarized in the Table 1.2.

Table 1. 2 Recently developed SPIONs for MRI.

T2 agent	Relaxivity (mM s)⁻¹	Side of Action	Size (nm)	Ref.
Resovist [®] (Carboxy dextran coated)	R1: 12 R2:188	Liver	62	[94]
Endorem [®] (Dextran coated)	R1: 24 R2:107	Liver	58	[91]
Sinerem [®] (Dextran coated)	R1: 23 R2:53	Liver	17-20	[97]
Polyethyleneglycol coated magnetoliposomes	R1: 3 R2:210	Bone marrow	40	[98]
Magnetoferitin (Protein coated)	R1: 8 R2:218	Blood pool	12	[99]
Ferumoxsil [®]		Gastrointestinal	200	[92]

Besides the commercial SPION based contrast agents, there are numerous SPION compositions developed with different coatings in various hydrodynamic sizes in the literature which are MRI active both in the *in vitro* and *in vivo* models. Strable *et al* synthesized SPIONs in polyamidoamine (PAMAM) dendrimers by the oxidation of Fe²⁺ in the size range of 20-30 nm. [100] Since, PAMAM coated SPIONs are highly stable in biological media and possess high R1 and R2 values, they are promising candidates for MRI contrast agents. One of the most important struggle in the use of nanoparticles currently is to design targetable iron oxide nanoparticles which accumulate mostly on the desired organ or tissue in order to enhance the contrast at the site of the interest and prevent accumulation of the nanoparticles in

healthy tissues. This would both improve the contrast, reduce the side effects and the necessary dose. Conjugation of an antibody specific to the antigen of the cell membrane receptor of the desired cell is a common approach in targeting nanoparticles to a specific cell. the. Artemov *et al* developed streptavidin conjugated SPIONs and coupled with biotinylated antiHer2 antibody to be specifically internalized by breast cancer cells overexpressing Her2-neu receptor. [101] With this way, they generated strong T2 contrast in breast cancer cells. In a similar approach, Josephson *et al* also achieved receptor guided MRI of hepatocytes. They conjugated the asialofetuin onto SPIONs which has affinity to the hepatic asialoglycoprotein and developed SPION based selective MRI probes. [102] In addition to the intrinsic MRI activity of SPIONs, multimodal imaging probes also can be generated using SPIONs such as fluorescence/MRI or positron emission tomography (PET)/MRI. Chakrawarty *et al* synthesized germanium 69 labelled SPIONs by chelator free approach. *In vivo* lymph node imaging was done by PEGylated ^{69}Ge coupled SPIONs in dual modality. [103] (Figure 1.7) Selective and dual mode imaging with SPIONs can be achieved due to functional coatings.

MR responsivity of SPIONs are further utilized in variety of applications from image guided therapy to bio-sensing. [104, 105]

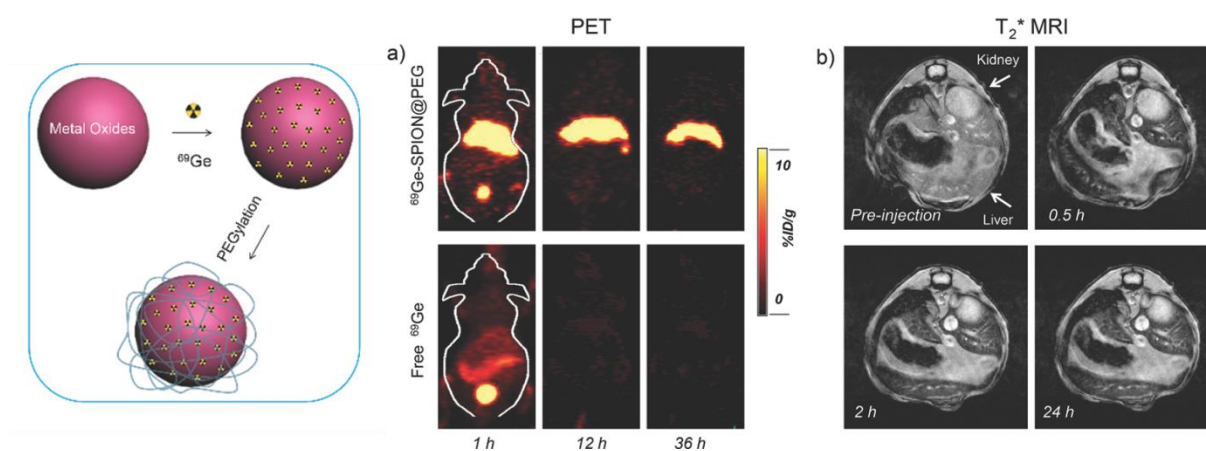


Figure 1. 7 Germanium 69 labeled SPIONs for PET/MRI. Schematic representation of the synthesis of ^{69}Ge labeled SPIONs with chelator free approach. (Left) (a) Serial in vivo PET

images of ^{69}Ge labeled SPIONs and free ^{69}Ge . (b) T2 weighed transaxial MRI images of ^{69}Ge labeled SPIONs after intravenous injection to mice showing accumulation on the liver rather than kidney. [103]

1.4.3. SPIONs in magnetic fluid hyperthermia

Magnetic fluid hyperthermia is a therapy method which is based on the tissue damaging ability of SPIONs by generating local heat. When SPIONs are subjected to an alternative magnetic field at low or moderate frequency, heat is evolved due to the hysteresis loss. In other words, under magnetic field oscillating behavior of the magnetic moments causes warming up the tissue in which SPIONs accumulate. The local temperature increase is related to the magnetic properties of SPIONs, strength of the alternating magnetic field, frequency of the oscillation and cooling capacity of the blood stream around the tumor tissue. The heating mechanism of SPIONs is explained by the Brownian and Néel relaxations which are the two fundamental paths for energy dissipation processes of single domain particles. Therefore, heating capacity of SPIONs is highly sensitive to their size, shape and crystal structures. [106, 107]

Since tumor cells are irreversibly damaged in between 42-45°C, magnetic hyperthermia is extensively applied for anticancer applications. It was investigated that, 30 mins local heating is sufficient to permanently damage tumor tissue. [106, 72] Hyperthermia can be applied using various formulations of SPIONs from capping agent coated iron oxide nanoparticles itself to magnetic nanoparticle loaded liposomes. Jordan *et al* demonstrated a successful hyperthermia therapy on a glioblastoma rat model for the treatment of invasive brain tumors. [72] Two kinds of SPIONs were analyzed to test their *in vivo* heating capacity : carboxy dextran coated SPIONs (mean diameter : 3 nm) and aminosilane coated SPIONs (mean diameter : 15 nm). Interestingly, carboxydextran coated SPIONs were unable to increase the

temperature higher than 39°C but provided an anticancer effect and improved the survival of the rat models. Aminosilane coated SPIONs were reported as much more successful than the former SPION. Autopsy analysis revealed that there is no iron deposits on the brain tumor after intra-tumoral injection of carboxydextran coated SPIONs. This result suggests that the surface chemistry of SPIONs is distinctive for the retention of SPIONs in the brain tissue. Ito *et al*, also reported an important result for the hyperthermia therapy on tumors. [108] They tested heating capability of cationic liposomes loaded with magnetic nanoparticles after intra-tumoral injection into MM46 mammary mice. Animals were exposed to an alternating magnetic field for 30 mins and the temperature was raised to 45°C in the tumor tissue resulting in damage. However, animals developed resistance to hyperthermia after repeated cycles. This was explained by the upregulation of heat shock proteins in the tumor tissue integrated with the immune cascade reactions.

Hyperthermia is also used in adjuvant chemotherapy. Within this aim, Ang *et al* reported heat triggered drug release using thermoresponsive polymer poly(N-isopropylacrylamide) (PNIPAm) around the iron oxide nanoparticle. [109] Release of anticancer drug was achieved above the lower critical solution temperature (LCST) of PNIPAm.

There are clinical studies, as well. Johannsen *et al* introduced aminosilane coated SPIONs to the patients suffering from prostate cancer by transperineal injection with 6 cycles. [110] They measured 41.1°C inside the tumor tissue. Although treatment exhibited an efficient anticancer activity, side effects such as urinary problems or tiredness were encountered in the patients. Aminosilane coated SPIONs were tested also on various patients having different neoplastic diseases and glioblastoma with local injections and control on tumor growth was observed during these treatments. [111, 112] Pre-clinical and clinical applications show that SPIONs provide a feasible strategy for hyperthermia.

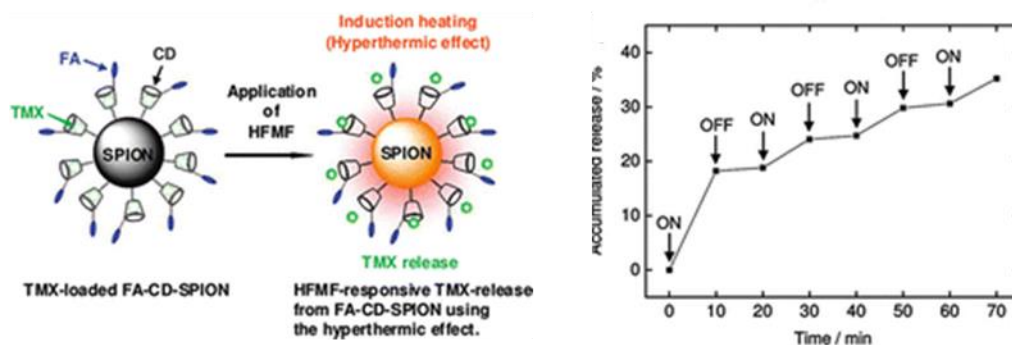


Figure 1. 8 Schematic representation of TMX release from FA-CD-SPIONs using the hyperthermia by applying a high frequency magnetic field (HFMF) (right) and controlled release of TMX from FA-CD-SPIONs by switching a HFMF on and off (left). [113]

Targeting magnetic nanoparticles to tumorous tissue for effective hyperthermia is also desired. Using targeting agents such as antibodies, peptides, citric acid or folic acid which are specifically targeting receptors overexpressed on the cancerous cells, magnetic nanoparticles can be directed to the desired tissue and invasiveness of hyperthermia on the neighbour healthy tissues can be significantly reduced. In this context, Hayashi *et al* loaded the hydrophobic drug tamoxifen as inclusion complex into the β -cyclodextrin (CD) moieties attached to the SPIONs. [113] Since CDs are decorated with folic acid, SPIONs are targeted to cancer cells more than healthy tissues. Hence, they achieved heat treated release of tamoxifen from CDs under alternating magnetic field. (Figure 1.8)

1.4.4. SPIONs in magnetically assisted drug delivery

Success of conventional drug therapies are generally limited by the poor enrichment of the drug at the diseased site. Hence, carrier mediated drug delivery is intensely studied to improve the pharmacodynamics and the pharmacokinetics of the therapeutics to enhance the drug efficacy and decrease their side effects.[114] Through the encapsulation of drugs into

biocompatible nano-carriers such as proteins, lipids, polymers, iron oxide nanoparticles, gold nanoparticles, their blood circulation time can be prolonged. [114] Since SPIONs are magnetically addressable, they can be selectively accumulated at the diseased site by applied magnetic field. This enriches the drug at the diseased site, enhance the drug efficacy and reduce the side effects of the payload. [115, 116] This becomes especially effective if the drug release is also triggered by the temperature increase created by hyperthermia. This allows entrapment of the drug until it reaches the destination and hyperthermia is created which would reduce the side effects dramatically and provide an adjuvant therapy[113] In the field of drug delivery, SPIONs smaller than 100 nm are generally suitable for hyperthermia while the ones larger than 100 nm are highly beneficial for magnetic targeting due to their better magnetic response.[117] SPIONs are available for drug delivery when designed as composite particles of magnetic core and a biodegradable inorganic or organic shellsuitable for drug entrapment. Hence, shell around the magnetic core is available for further chemical modifications. [118]

Hydrophobic drugs are difficult to administer since blood is aqueous. Loading of *hydrophobic* drugs in nanoparticles are generally managed by two different methods. (1) Nano-precipitates can be prepared with the addition of the dissolved drug and shell materials in a water *miscible* organic solvent into the aqueous SPIONs followed by evaporation of the organic solvent. [119] (2) nano-emulsions can be produced by addition of the dissolved drug and shell materials in a water *immiscible* organic solvent into the aqueous SPIONs with a stabilizer. [120] *Hydrophilic* drugs also can be loaded to SPIONs through the strong electrostatic interaction between the drug molecule and the shell matrix around the magnetic core. [121] Most significant consideration for the loading of hydrophilic drugs is to prevent the burst release which may result in a pharmacokinetic profile similar to the free drug. Therefore, the

affinity of the drug molecule to the surface ligand of SPIONs has to be tested by drug release analysis.

In order to achieve magnetic targeting with SPIONs, magnetic susceptibility of nanoparticles has to be sufficient whereas the applied magnetic field has to be strong enough to ensure directing them onto the site of action before releasing their payload. Release mechanism of the drug on the targeted tissue can occur by diffusion from the matrix around the magnetic cores, cleavage of the drug-nanoparticle bond or by the degradation of the matrix triggered with pH, temperature or enzymatic reactions. [122-124] In case of the applications of magnetically targeted systems, acting distance and the position of the external magnet has major impact on the therapeutic efficiency. [125, 126] Generally permanent neodymium-iron-boron magnets are used in combination with SPIONs. [127] Or transplanted magnets generate much effective magnetic field over the selected biological compartment in the body. Pacheco *et al* reported a study about the targeted release of Doxorubicin from iron-carbon nanoparticles in the kidney containing a magnetic transplant. [128]

Capability of SPIONs as drug delivery vesicles is extensively studied pre-clinically and their magneto-responsivity related therapeutic efficiency are demonstrated in various studies. For instance, Gang *et al.* achieved a perfect therapeutic improvement in the action of Gemcitabine loaded into SPIONs with emulsion technique. [129] They oriented nearly 150 nm SPIONs magnetically into pancreatic tumor of the mice model and detected an important antitumor property even at the 15 fold lower doses of Gemcitabine. In a similar manner, Yu *et al* load Doxorubicin onto PEGylated SPIONs electrostatically and directed nanoparticles into lung carcinoma of the model mice. [130] They observed remarkable accumulation of the nanoparticles on the tumor site with nearly 8 fold lower dose along with dramatically lower level of drug in the non-targeted tissues.

Besides the magnetic accumulation, tissue specific targeting is also widely used method in combination with SPIONs. Various ligands from different categories are attached on the matrix around the SPIONs to gain affinity towards the desired tissue and combinatory targeting can be achieved. For instance, since ovarian carcinoma cells and squamous carcinoma cells overexpress folate receptors, SPIONs conjugated with FA are widely used to target related tumors and to deliver anticancer drugs selectively to tumors. [131]

1.4.5. SPIONs in magnetofection (Gene delivery)

Magnetofection is a biomedical term corresponding delivery of double or single stranded nucleic acids at different base pairs into the cytoplasm or nucleus of the target cells under magnetic assistance. Since magnetically responsible bodies are needed to direct genes into the side of action, magnetofection is mostly conducted with SPIONs.[132] Therapeutic nucleic acids can be deoxyribonucleic acids (DNAs), micro ribonucleic acids (miRNA) or small interfering ribonucleic acids (siRNAs).[133] The most significant biological barrier in the gene delivery is the high susceptibility of nucleic acids to enzymatic degradation, limited cellular internalization due to their negative charge and hydrophilicity.[134] Therefore nucleic acid based therapeutics have to be administrated with a carrier based delivery strategy. In the preclinical and clinical applications, there are various transduction (gene delivery by viral carriers) and transfection (gene delivery by non-viral carriers) studies. Nucleic acids can be incorporated into nano-carriers by electrostatic interaction or entrapment within the matrix. [135] SPIONs coated with a polycationic matrix are successfully capable of magnetically active transfection of the therapeutic genes into the concerned cells. The necessity of polycationic organic shell is arising from the strong electrostatic interaction between negatively charged nucleic acids and the positively charged shell.[136] Polyethyleneimine (PEI) is a widely used polycation in the transfection studies because it has large number of secondary amines which provides strong interaction with the nucleic acids and lysosomal

escape with the “proton sponge mechanism”. [137] PEI is used to generate magnetically active composites in combination with SPIONs for the preclinical transfection studies. Mykhaylyk *et al* synthesized PEI coated SPIONs and show their magnetofection efficiency using green fluorescent protein (GFP) reporter gene on HeLa cells.[138] Hirao *et al* developed a carrier system for eGFP genes from the magnetically active cationic liposomes.[139] Magnetic liposomes were generated from 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and 1,2-dioleoyl-3-snphosphatidyl ethanolamine (DOPE) molecules. Nanoparticles were incubated with osteoblasts and He99 lung tumor cancer cells with or without applying an external magnetic field. It was observed that transfection efficiency of the magneto-liposomes is nearly 7 times higher under magnetic field compared to the condition lacking magnetic field. Magnetofection on stem cells is also widely studied to modify their self-renewal and the proliferation properties. Lee *et al*, transfected eGFP gene to murine embryonic cells with SPIONs and they obtained much higher transfection efficiency (45%) compared to standard gene transfection agent FuGENE.[140] Besides, transfection of eGFP was sustained during the stem cell proliferation. This shows that, magnetofection can be effectively applied to the embryonic stem cells.

Although magnetofection is widely studied in vitro applications, there are much less in vivo studies. Scherer *et al*, send fluorescently labeled genes on the surface of the SPIONs by the injection on the femoral artery of the model mice.[141] They observed a remarkable fluorescence on the cremaster muscles of the testes under magnetic field compared to lack of magnetic field which shows an effective magneto-oriented delivery of the gene of interest for *in vivo* models.

Apart from their extensive use in MRI, magnetic hyperthermia, drug and gene delivery, SPIONs are also emerging in the fields of tissue engineering [142], cell separation [143], cell

labeling [144], photodynamic therapy [145], radioimmuno therapy [146], magnetic relaxation switches [147], immunoassays [148], enzyme immobilization [149].

1.5. The Proposal and the Aim of the Thesis Work

SPIONs are widely used in pre-clinical and clinical applications due to biocompatibility of the iron oxide, possibility of synthesizing them with different surface coatings allowing them to be loaded with a payload and even conjugated with targeting ligands, fluorescent dyes or even with other nanoparticles. Despite of all the studies in medical use of SPIONs, especially in the gene/drug delivery, molecular targeting, combination therapy, there are still significant problems limiting the emergence of new commercial products. These include the toxicity of the coating material, inability to track particles *in vitro* or *in vivo* without MRI (which is not easy for every research group), large aggregate sizes or aggregation after surface functionalization or conjugation protocols and/or insufficient amount of payload binding, release or stability, and finally their targeted delivery to the site of interest due to many problems including large sizes, insufficient ligand conjugation or interaction with the target, or intratumoral heterogeneity, even insufficient magnetic field for magnetic dragging.

It is highly desired to develop SPIONs that can deliver therapeutic oligonucleotides to tumor selectively in therapeutic doses, with non-toxic SPIONs which can be tracked with dual mode, specifically optically and in MRI. For selective molecular targeting, SPIONs have to be smaller than 150 nm so that they can circulate in the blood long enough before taken up by RES. For dual mode imaging they need to be conjugated with a fluorescent entity. Being non-toxic is the most difficult task since anionic oligonucleotides require cationic coatings to bind and cationic nanoparticles are quite toxic.

The goal of this thesis work is to develop small SPION based nanoparticle portfolio with improved biocompatibility, optical imaging modality and high oligonucleotide transfection

efficiency which can eventually target tumor cells despite of their heterogeneity. Such particles would be excellent multifunctional particles with the potential of dual imaging (MRI and optical) , dual targeting (molecular + magnetic) and potentially with combination therapy potential (gene + hyperthermia + drug, or any combination of these). Yet, it is not an easy task and these problems were attached in different ways as summarized in different chapters somewhat separately.

In order to gain multi modal imaging capability to SPIONs, iron oxide nanoparticles and fluorescent systems such as quantum dots or organic dyes have been combined in the different ways. However the overall hybrid structure usually deteriorates some of the quality of each component such as loss of luminescence intensity, aggregation of SPIONs, reduction in surface functional groups and most importantly they may increase the toxicity such as in case of combination with quantum dots or luminescent silica nanoparticles. Dyes are usually less toxic, but suffer from short self-life , photo-bleaching and quenching since SPIONs are strong absorbers in the visible region In order to overcome these limitations, a feasible design strategy with the magnetic nanoparticles has to be developed to gain multi-functionality by minimum chemical modifications. In chapter 2, development of dye free fluorescent iron oxide nanoparticles as multifunctional theranostics nanoparticles for dual mode imaging and gene therapy will be discussed. It was discovered in this thesis work that poorly luminescent polyethyleneimine (PEI), which is the most widely used coating for nanoparticles designed for gene transfection, become strongly luminescent when adsorbed on the SPION crystals during the synthesis allowing the optical tracking of these SPIONs *in vitro* without a need for a dye or another luminescent species. Microscopic and macroscopic analysis of the core and the coating and investigation of the phenomenon to the synthetic method are proposed for elucidation of the origin of this phenomenon and Chapter 2 does summarize all these efforts and the finding. Dual mode imaging and gene transfer ability of PEI coated iron oxide

nanoparticles were shown by biomedical tests. This finding about bright and *in vitro* detectable intrinsic luminescence of SPIONs moves the diagnostic capabilities of magnetic nanoparticles one step further from the MRI. Designing a luminescent model system which combines the iron oxide nanoparticles and PEI, does not only provide a facile approach for the generation of multifunctional nano-systems, but also significantly contribute to the literature of luminescent polyamines by investigating the origin of the poor luminescence coming from these polymeric systems.

Toxicity is another most limiting factor in the applicability of multifunctional SPIONs for gene delivery due to the required cationic coatings, which is mostly PEI. Even though several methods have been developed to decrease the toxicity of gene delivery agents such as introducing biocompatible polymers (PEG) or transfection with neutral and anionic bridged lipid carriers, these procedures generally decrease the gene loading capacity or blood circulation time of the delivery vesicles. For this reason, there is a fundamental need to produce more versatile gene delivery agents which exhibit sufficient protection, efficient transfection with minimal toxicity and reduced affinity to the phagocytic system at the same time. One very important finding reported recently, shows that intercellular microRNAs are carried by a member of Argonaute protein family (Argonaute 2) in the living organisms. This is indeed a revolutionary finding stating that proteins actually bind and protect micro-RNA in the blood. This brings out a unique idea of “a natural miRNA carrier” that can be developed outside of the body and deliver the miRNA to the body. Inspired by miRNA carrying capability of Argonaute 2 in extracellular media, Argonaute 2 (AGO2) protein bound SPIONs are proposed as a new generation miRNA delivery vehicles. With this motivation, multifunctional SPION bio-conjugates with fluorescent dye conjugation to allow optical tracking and which are able to target breast cancer via antiHER2 conjugation are proposed. As a target delivery of an autophagy related therapeutic miR374b to breast tumor was offered in

this thesis. Besides, magnetic dragging as a second targeting mechanism, hyperthermia generation as a second mode of therapy and even combination therapy with cisplatin in the *in vitro* and *in vivo* breast cancer cells and tumors are proposed. This study was supported by TUBITAK and conducted with the collaboration of Prof. Devrim Gözüaık and Prof. Ali Koşar groups from Sabanci University. Chapter 3 summarizes all the critical steps in the synthesis of such particles which showed a very good biocompatibility, targeting efficiency and miRNA delivery to the cancer cells and tumors.

Another significant issue in the biomedical applications of the various nanoparticles such as SPIONs is poor molecular targeting strategies which are only focused on a single overexpressed cell signaling receptor of cancer cells. However, cancer, especially breast cancer, is a dramatically heterogeneous disease with different phenotypes and expression abnormalities. Therefore, more strong targeting strategies have to be developed to involve all possible cancer cell signals. In order to overcome the drawback about the targeting breast cancer cellular heterogeneity, development of SPIONs capable of targeting both human epidermal growth receptor 1 (EGFR) and 2 (HER2), is proposed in this thesis. In order to track them optically targeting ligand were tagged with fluorescent dyes which emit at different wavelengths. Chapter 4 discusses the approach and the synthesis of monospecific (only HER2 targeted, only EGFR targeted) and bispecific (both HER2 and EGFR targeted) SPIONs designed for breast cancer cells lines containing different HER2 and EGFR expression levels. This approach is an important step forward to enhance specific delivery of SPIONs and its therapeutic payload later to the tumor with greater efficiency which would reduce the dose requirement and the side effects.

Overall, the aim of this thesis is to contribute to the literature of theranostic iron oxide nanoparticles through the novel designs and compositions, and enhance the scope and the utility of SPIONs as very promising theranostic nanoparticles in clinical applications.



CHAPTER 2

2. SYNTHESIS AND CHARACTERIZATION OF SELF LUMINESCING PEI-SPIONs FOR THERANOSTIC PURPOSES

2.1. Introduction

Luminescent materials are of great interest for biotechnology and medicine since they can be utilized in high value applications such as sensors, labeling, and imaging.[150-152] Luminescent proteins, synthetic polymers and quantum dots are the most popular luminescent materials with their own advantages and disadvantages.[153-155] Luminescent polymers are generally π -conjugated systems with delocalized electrons or contain fluorophores as pendent or end group.[156, 157] Conjugated polymers usually suffer from insolubility which limits processability and hence, the medical use.[158, 159] However, in recent years, amine containing branched polymers such as polyethyleneimine (PEI) [160-164] and dendrimers such as polyamidoamine (PAMAM) [165-169] are reported as weakly blue luminescent organic materials. Although the intrinsic mechanism of this luminescence is still not clear, several speculations have been reported. Larson et al suggest $n \rightarrow \pi^*$ transition as the origin of PAMAM luminescence and indicated that dense packing enhances such luminescence. [170] Lin et al. claims that the luminescence of PAMAM is originating from the N-OH formed as a result of oxidation [167] Lee et al. showed that oxidized PAMAM is the real luminescent form.[166] Imae et al suggested that interaction of O_2 or oxygen containing molecules with internal tertiary amines forming exciplex is the origin of blue luminescence. [165, 169] They also showed that luminescence of PAMAM dendrimers increase dramatically with protonation, especially below pH 6 where tertiary amines get protonated. [168] Although they are not sure about the origin of the luminescence itself, influence of the pH was

explained with increasing charge-charge repulsion and enhanced H-bonding. Pastor-Perez *et al* studied the luminescence of linear and branched PEI. [163] They showed that blue emission obtained from linear PEI with no tertiary amines is dramatically stronger than the luminescence of branched PEI. Yet, methylation, oxidation or acidification, all increased the quantum yield of both linear and branched PEI with molecular weight between 1.8 to 15kD. The origin of the blue luminescence was attributed to the creation of amine rich nanoclusters and electron-hole recombination processes. Pan *et al* has shown that formation of carbamate groups via CO₂-amine reaction causes a strong blue luminescence of PEI. [162] When all related data and suggestions are considered, it seems like there is not a single mechanism that explains the intrinsic fluorescence of these “periodically amine” containing species. Different factors including delocalization of nonbonding electrons in highly repeating systems, rigidity of backbone, acidification of amines, hydrogen bonding, exciplex formation, amine oxidation and solvent induced aggregation were reported as factors that amplifies the weak luminescence of PEI and amine containing dendrimers.[160, 165, 161-163, 169]

However, luminescence of these materials are especially valuable since they are widely used for drug and gene delivery and may provide label-free tracking of these agents *in vivo* and *in vitro*. [171, 172] Although both PEI and PAMAM have been studied for drug/gene delivery for decades, these systems have not been recognized as luminescent delivery vehicles due to very weak luminescence until recently. Lin *et al* synthesized mostly linear PEI and demonstrated siRNA delivery with this luminescent polymer.[173]

Sun *et al* utilized the luminescence of branched PEI as the imaging modality in polymeric nanoparticles (PDOTs) formed from amphiphilic polyethyleneimine-poly(lactide) (PEI-PLA).[164] Enhanced luminescence of the PEI in the PDOT (quantum yield=0.31) compared to free PEI (quantum yield=0.01) was attributed to the more compact structure of the PEI in

the self-assembled PDOT. Actually, this was the first report which studied the luminescence of branched PEI at relatively high molecular weight (25 kDa).

Cationic polymer PEI, especially branched PEI at 25kDa molecular weight, has been accepted as the golden standard of non-viral nucleic acid delivery vehicle providing efficient binding to the cell surface, endosomal release of the cargo and translocation to the nucleus. [174, 171, 175, 176] In an effort to develop theranostic nanomaterials, PAMAM and PEI were coupled with superparamagnetic iron oxide nanoparticles (SPIONs) for drug/gene delivery combined with magnetic resonance imaging.^{[177, 178] [179]} Usually, these systems were conjugated with other fluorescent tags for the optical detection of the particles in the cell in many *in vitro* studies including, for example, flow cytometry or fluorescence imaging, since its blue luminescence is weak and most probably not recognized .[163] Theranostic materials combining therapeutic and diagnostic moieties are highly desired in medicine in order to track the destiny and detect the outcome of therapy. There are also examples to luminescent SPIONs in which quantum dots (QD) were used as the luminescent entity.[180-183] QDs are considered more advantages than dyes since they have much longer luminescence lifetime, broad absorption and narrow emission bands.[181, 182] Such QD-SPION hybrid structures are of great interest due to multi-functionality including dual imaging (optical imaging and magnetic resonance imaging), imaging and magnetic targeting, diagnosis and hyperthermia or combined therapy.[66, 184, 185] Yet, toxicity of QDs is usually a significant concern. An important issue also is the fact that attaching fluorophores (dye or QD) that are active in the visible range onto iron oxide surface, usually results in reduction or quenching of luminescence [182, 186][182, 186][182, 186][182, 186][182, 186][182, 186][183, 187][183, 187][183, 187] since SPIONs have a strong absorption in the UV and visible range of the spectrum. However, an enhanced luminescence was also reported for a dye conjugated SPION and it was attributed to the photo-induced charge transfer. [187]

A nanoparticle composed of biocompatible superparamagnetic iron oxide and blue luminescent cationic polyethyleneimine would be very interesting theranostic nanoparticle capable of gene transfection, magnetic and optical tracking/imaging and potentially provide dual therapy, namely gene therapy and hyperthermia. The greatest advantage of such material is the elimination of the need for fluorescent tagging with a dye or QD which makes the synthesis simpler and cheaper and the composition safer and better. The former fluorescent tag quenches very quickly limiting operational time and the latter is a relatively expensive and potentially a toxic tag. Besides, each functionalization reduces the number of available amine groups which in turn reduce gene binding ability.

Here, we report a novel luminescent-superparamagnetic gene delivery system based on PEI coated SPION nanoparticles exhibiting an exceptionally strong blue luminescence. Conditions affecting the luminescence intensity of PEI and the SPION/PEI system and the origin of luminescence were investigated. Polyinosinic: polycytidylic acid (poly I:C), an important immune-suppressor and synthetic analog of double stranded RNA [188-190] was electrostatically loaded onto these magnetic and luminescent SPION/PEI nanoparticles to demonstrate the potential of the nanoparticle as a therapeutic agent. Loading efficiency, cytotoxicity, potential as optical and magnetic imaging agents was discussed to evaluate the suitability of these multifunctional nanoparticles in cancer treatment and imaging applications.

2.2. Experimental

2.2.1. Materials

$\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ salts were purchased from Merck (U.S.A.). 25kDa branched PEI and ammonium hydroxide (26 %) were purchased from Aldrich (U.S.A.). Hydrochloric acid (37 %) was purchased from Carlo Erba (France). Acetic acid (99.8 %) was purchased from Lachema (Czech Republic). Agarose gel was purchased from Invitrogen (U.S.A.).

Polyinosinic-polycytidylic acid sodium salt (poly I:C) was purchased from Sigma-Aldrich (U.S.A.). Tris Borate EDTA (TBE) buffer was purchased from Alfa Aesar (U.S.A). Dubelcco's Modified Eagle's Medium (DMEM) with 4.5 g/L D-Glucose, L-Glutamine and Pyruvate was purchased from Life Technologies (U.S.A.). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was purchased from Appllichem. HeLa cell line was obtained from Dr. Devrim Gozuacik at Sabanci University (Turkey). 96-well plates were purchased from Greiner Bio-one (Austria).

2.2.2. Synthesis of Branched PEI (bPEI) Coated SPIONs (bPEI-SPION)

0.07 M $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ and 0.14 M $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ salts were mixed with 0.6 mM bPEI at room temperature under Argon and treated with 4.5 M NH_4OH at 80 °C. After 30 min reaction, colloidal solution was cooled to room temperature and sit atop a magnet overnight to remove any large aggregates if any exists. Then, the colloidal solution was acidified to pH 5 either with 6.5 M HCl or 8.7 M CH_3COOH and set aside for 3 h. It was then washed with DI water using 30 kDa (molecular weight cut off) Amicon centrifugal filters to remove unreacted species.

2.2.3. Preparation of PIC loaded bPEI-SPIONs (bPEI-SPION/PIC)

PIC was dissolved in nuclease free water to a final concentration of 10 mg/mL as stock. In order to make ds PIC, it was heated to 55 °C then cooled back to the room temperature according to manufacturer's instructions (Aldrich). 500 µg/mL of PIC in HBG (HEPES buffered glucose: 20 mM HEPES, 5 % w/v glucose, pH 7.4) and 500 µg/mL of bPEI-SPIONs in HBG were mixed to generate bPEI-SPION/PIC in HBG at a final nucleotide concentration of 100 µg/mL. bPEI-SPION and PIC were incubated at room temperature for 30 minutes at different ratios (0.5:1, 1:1, 1:2, 1:4, 1:6, 1:8) and thereafter, binding efficiency was checked with gel electrophoresis. For this, 1.2 g of agarose was dissolved in 100 mL tris borate EDTA

buffer (TBE) buffer and boiled 100 °C. After cooled down to 70 °C, 4 µL ethidium bromide was added and the gel was poured onto gel electrophoresis unit. 50 µL of the solution to be analyzed were mixed with 5 µL of DNA gel loading buffer solution and inserted into the gel wells and separated under electric field (80 mV, 400 mA) for 60 min with Bio-Rad Mini Sub Cell GT Cell.

2.2.4. Cytotoxicity Assay of bPEI-SPION and bPEI-SPION/PIC

HeLa human ovarian cancer cells were grown in 75T culture flasks in DMEM culture medium supplemented with 10 % FBS, 1 % penicillin solution at 37 °C under 5 % CO₂. They were sub-cultured three times per week with 0.25 % trypsin-EDTA. Anti-proliferative effects of free bPEI, bPEI-SPION, bPEI-SPION/PIC on HeLa cells were evaluated by MTT cell proliferation kit according to the manufacturer's instructions. Cells were seeded on to the 96 well microtiter plates (Greiner) at a concentration of 5×10^3 cells/well and incubated for 72 h in medium. In each plate, assay was repeated for the blank medium and untreated cells in medium as controls. Then MTT reagent was added to each well and soluble formazan product was measured at 600 nm with Biotek ELx800 96-well plate-reader. Results were reported as the average of 3 replicates.

2.2.5. Fluorescence Imaging of bPEI-SPION and bPEI-SPION/PIC

HeLa cells in 24-well plates were incubated with free bPEI, bPEI-SPION and bPEI-SPION/PIC at a constant 10 µg/mL bPEI content. After 2 hours incubation at 37 °C, medium was removed and washed with PBS for several times. Cell nuclei were stained with DAPI according to manufacturer's instructions. Fluorescence images were obtained with Olympus-excellence RT Life Science Microscope ($\lambda_{exc} = 358$ nm and $\lambda_{emm} = 485$ nm).

2.3. Characterization Methods

Photoluminescence measurements were performed with Horiba Jobin Yvon FluoroMax-3 at room temperature. Quantum yield (Φ) of aqueous bPEI-SPIONs was calculated using the following standard equation using anthracene in ethanol as the reference ($\Phi=0.27$).

$$\Phi(\text{sample}) = \Phi(\text{reference}) \times \frac{\text{Grad}(\text{sample})}{\text{Grad}(\text{reference})} \times \frac{\eta(\text{sample})}{\eta(\text{reference})}$$

Five different concentrations of the reference and the sample with absorbance below 0.15 at the excitation wavelength of 355 nm were measured and plotted as intensity versus concentration to drive the $\text{Grad}_{\text{sample}}$ and $\text{Grad}_{\text{reference}}$.

Fluorescence life time measurements were done by Horiba Fluorolog equipped with TCSPC Triple Illuminator. 456 nm Horiba Nanoled pulsed diode light source (pulse duration 1.2 ns) is used. Measurement time 100 ns was taken for 4096 channel.

Absorbance data were collected with UV-3600 Shimadzu UV-VIS-NIR spectrophotometer at room temperature. Dynamic light scattering (DLS) experiments were conducted with Zetasizer Nano Series ZS at room temperature with 173° backscattered angle. Functional groups were investigated using the dried samples and Thermo Scientific Nicolet iS10 ATR-IR. Transmission electron microscopy (TEM) images were taken using Techani G2 F30 at brightfield high resolution (HR) TEM (acceleration voltage 200 kV using samples deposited on carbon coated Cu-grid from dilute solutions. Atomic Force Microscopy (AFM) micrographs were obtained by Bruker Dimension Ikon at ScanAsyst mode scanning with ScanAsyst-Air tip. Samples were covered on mica from dilute solutions for AFM studies. Vibrating sample Magnetometry (VSM) measurements were conducted with Cyrogenic limited PPMS at ambient conditions. MRI images were taken with Siemens Magnetom Essenza 1.5 T MRI system with T_1T_S/T_2T_S MRI sequence at Via Hospital (Istanbul-Turkey).

X-band (9.86 GHz) continuous wave electron paramagnetic resonance (CW-EPR) measurements were performed with Bruker EMX spectrometer. We have used super high-Q (X-band) resonator from Bruker. The offset in the magnetic field and the accurate g-factors in X-band continuous wave measurements were determined with a polycrystalline DPPH (2-diphenyl-1-picrylhydrazyl) reference sample with well-known g-factor ($g = 2.0036$). Additional EPR spectral analysis has been performed by the aid of the WINEPR program from Bruker.

2.4. Results and Discussions

2.4.1. Synthesis and Characterization of bPEI-SPIONs

Blue luminescent bPEI-SPION nanoparticles were prepared via *in situ* coating of SPIONs with 25 kDa branched PEI (bPEI) via co-precipitation of iron salts in aqueous medium at 85 °C under basic conditions. After simple processing steps consisting of acidification and washing with DI water, colloiddally and optically stable magnetic and luminescent colloidal suspension of cationic nanoparticles was obtained. (Figure 2.1)

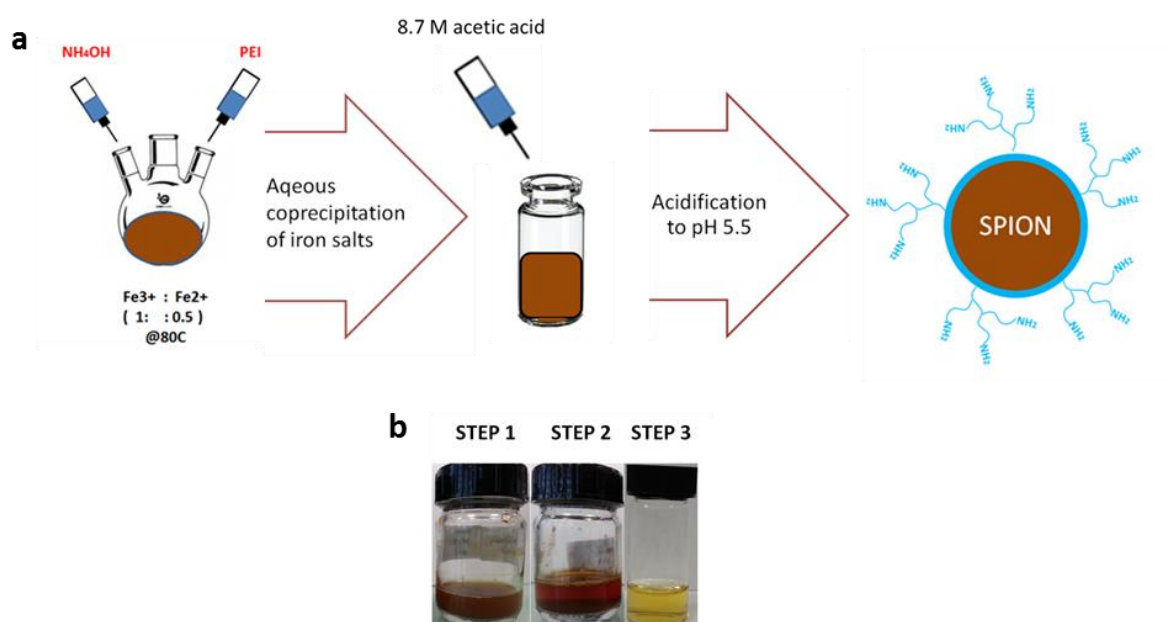


Figure 2. 1 a) Schematic representation of the synthesis of luminescing bPEI-SPIONs. **b)** Photographic representation of the preparation process of bPEI-SPION. Step 1: coprecipitation in water, step 2 : acidification (final pH=5), step 3: washing and dilution (10 mg /mL)

Cationic nature of bPEI-SPIONs was confirmed with zeta potential measurement: +18 mV. Size distribution of the particles was measured in different ways. Hydrodynamic size which includes the inorganic core and the hydrated polymeric shell measured by DLS is 30 nm (Figure 2.2 A). Overall particle size measured by AFM operated in magnetic mode is 20 nm (Figure 2.2 B). Average size of the inorganic core (SPIONs) was measured as 10 nm from the TEM images (Figure 2.2 C).

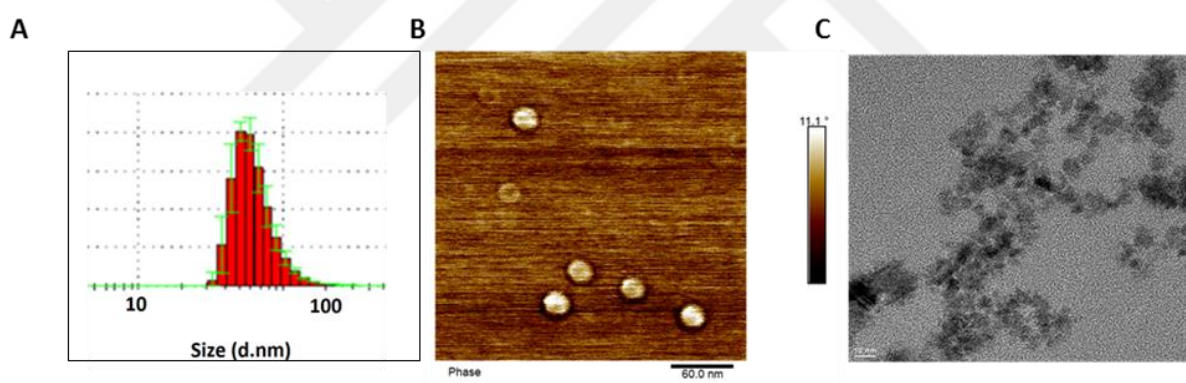


Figure 2. 2 (A) Hydrodynamic size measured by DLS and reported as the number average, (B) AFM micrograph, lift mode phase image (magnetic mode, 60 nm scale bar) (C) TEM image (10 nm scale bar) of bPEI-SPIONs.

PEI content of bPEI-SPIONs is a significant parameter to compare fluorescence properties of nanoparticles with this much amount of free bPEI, therefore amount of bPEI on bPEI-SPIONs was detected using thermogravimetric analysis (TGA) under inert atmosphere (Figure 2.3)

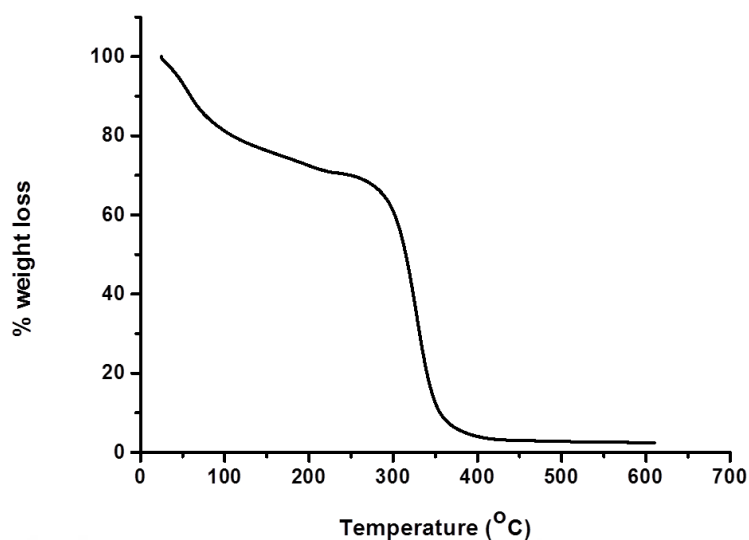


Figure 2. 3 Temperature dependent weight loss of bPEI-SPION after acidification with acetic acid.

2.4.2. Luminescence Properties of bPEI-SPIONs

bPEI-SPIONs show a strong blue luminescence with peak maxima at 485 nm when excited at 365 nm (Figure 2.4). Such strong emission is detected best after the usual work-up which involves acidification, washing and dilution steps (10 mg nanoparticle/mL) of the as-made nanoparticles. When acidified with HCl or acetic acid (AcOH) to pH 5, the luminescence intensity was improved 1.6 and 2.8 times, respectively, compared to un-acidified nanoparticles (Figure 2.4 a). Free bPEI solution shows blue emission with a peak maximum at 460 nm, in agreement with literature [163], but only when it is concentrated, for example at 13.3 mg/mL, which was the PEI concentration used in the SPION synthesis (Figure 2.4 a). TGA data shows that, aqueous suspension of luminescent bPEI-SPION contains 0.03 mg/mL bPEI. Free PEI at such low concentration lacks any detectable luminescence (Figure 2.4 b). Yet, at the same bPEI concentration (0.03 mg/mL) luminescence intensity of acidified bPEI-SPION is nearly 1200 times stronger than the free bPEI (Figure 2.4 b). Actually, at such low concentrations, free polymer looks indifferent than plain water under the UV light (Figure 2.4

b, inset). These indicate that deposition of PEI on to the surface of iron oxide nanocrystals dramatically enhances its luminescence with a small red shift of emission maxima to 485 nm. This enhancement in the luminescence intensity is quite unexpected, since SPIONs have a strong absorbance in the visible spectrum and hence, suggests poorer luminescence on the contrary to the observed effect.

Quantum Yield (QY) of acidified (pH 5) free bPEI is 1.3 % and bPEI-SPION is 3.3 % with respect to anthracene (absolute QY in ethanol=0.36). However, these QY values should be evaluated as relative values for the sake of comparison, keeping in mind that since the luminescence of free PEI is poor, in order to obtain a significant signal intensity, a highly viscous, concentrated solution (0.13 mg/mL) was used, which improves the emission intensity by restricting vibrational and rotational motions; and SPIONs have a strong absorption at both the excitation and emission wavelengths.

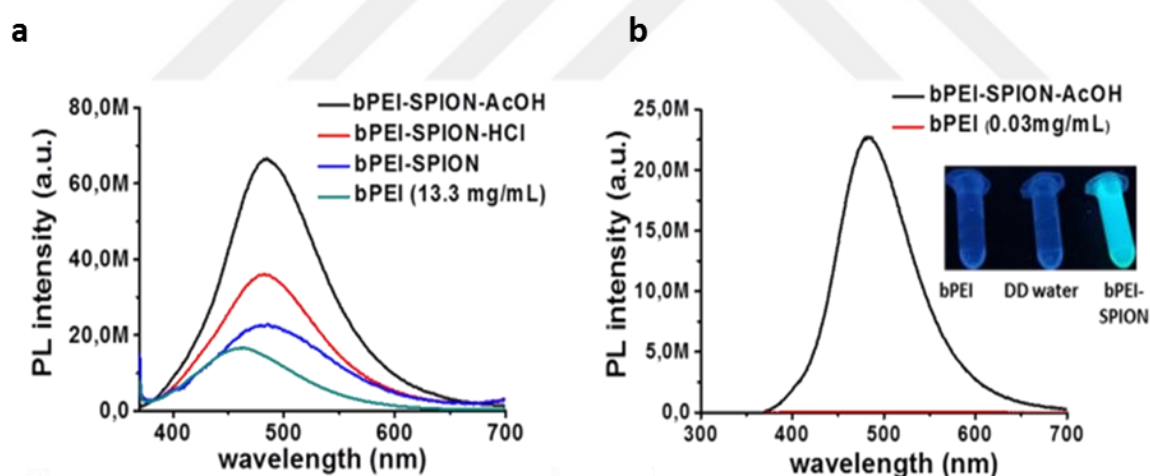


Figure 2. 4 The optical properties of bPEI and bPEI-SPIONs (a) Absorbance calibrated PL spectrum (excitation at 360 nm) of free bPEI (13.3 mg/mL), bPEI-SPION (10 mg nanoparticle/mL) before and after acidification (to pH 5) with AcOH, (b) free bPEI (0.03 mg/mL) and bPEI-SPION-AcOH (10 mg nanoparticle/mL containing 0.03 mg bPEI/mL). Inset: Picture of the bPEI (0.03 mg/mL), doubly distilled water and bPEI-SPION (containing 0.03 mg bPEI/mL) under UV-light (364 nm).

pH dependent enhancement in the luminescence of both methylated and non-methylated branched and linear PEI was investigated by Pastor-Perez et al.[163] Especially, methylated linear PEI shows a sharp increase in quantum yield from $\phi=0.02$ to $\phi=0.1$ when pH was reduced from 11 to 2. A similar pH dependent luminescence was also reported for PAMAM, poly (propylene imine), poly(amino ether) dendrimers. [168, 169, 191] Enhanced chain rigidity due to charge repulsion and enhanced radiative decay in bPEI at low pH are some of the proposed explanations to the improved quantum yield at acidic pH. [192] It is important to note that all of these studies used lower molecular weight PEI. Actually increasing luminescence intensity with increasing molecular weight has been reported as well and was rationalized with the requirement for cluster formation with high amine content. [160]

In order to study the pH effect thoroughly in our system, both free bPEI 25kDa and bPEI-SPION, which was initially adjusted to pH 5 with HCl and HAc, separately, and washed, were readjusted to pH 3.5, 7 and 11. In case of free bPEI, although absorption peak maxima did not change with the pH, at pH 11, where the polymer is deprotonated, absorbance decreased dramatically and a small blue shift was observed in the PL maximum (Figure 2.5 a). Emission intensities at pH values between 5 and 11 were similar but at pH 3 it was about 5 times stronger (Figure 2.5 b). This is in line with the improving luminescence of PEI with acidification. On the other hand, no significant change in the absorbance or fluorescence spectra of bPEI-SPION was observed with further pH adjustments (Figure 2.5 c-d). So, when the bPEI itself is considered, the enhancement in the emission intensity by acidification is about 5 times but this is incomparable with the enhancement achieved by deposition of the PEI on the SPION surface which enhanced luminescence 1200 times over the free PEI at same concentration (Figure 2.4 b). This, at least partially, may be attributed to the reduction of the non-radiative relaxation events due to the more rigid conformation of the bounded bPEI, somewhat analogous to the restricted mobility of PEI in the case of PEI-PCL PDOT

nanoparticles.[164] Besides, when delocalization of lone pair electrons of amine groups are considered, the distance between the amine groups in polymeric or dendritic structures may have a critical importance for the emission intensity. Cao *et al*, pointed out that dendrimers synthesized from ethylene diamine have stronger emission than the ones synthesized from 1,6 hexane diamine due to close proximity of the amine groups and enhanced delocalization of electrons. [160]

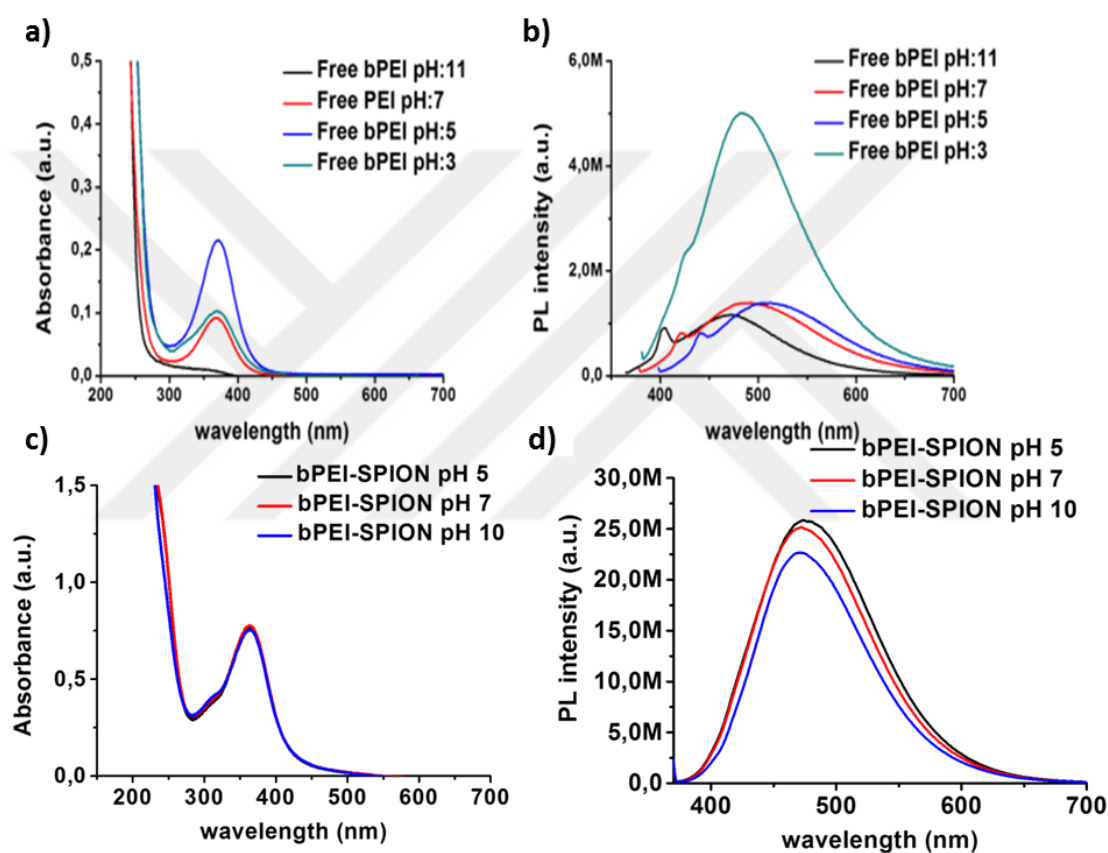


Figure 2. 5 Effect of acidification on free bPEI and bPEI-SPION. (a,b) Absorbance and fluorescence spectra of free bPEI (5 mg/mL) and (c, d) Absorbance and PL spectra of bPEI-SPION (0.03 mg/mL bPEI on SPION) at different pH values adjusted with AcOH.

Similarly, in the case of bPEI-SPION, adsorption of polymer on a rigid crystal surface creates a more compact arrangement for bPEI, closer proximity between amine groups which may contribute to the enhancement of the luminescence. Although acidification did not cause a significant change in the emission peak position, adsorption of bPEI onto the SPION surface

resulted in a red shift in the luminescence maxima from 460 nm to 485 nm. The former may indicate that not the type but the amount of the radiative events change with the acidification. The later may be the result of a changed bandgap or polarity. Indeed, the luminescence wavelength of bPEI-SPION shows an excitation dependent behavior. As the excitation wavelength increased from 355 to 475 nm, emission maxima shifted from 480 to 570 nm (Figure 3A). This means by changing the excitation wavelength it is possible to get blue, green or yellow luminescence from these particles. Dependence to excitation wavelength is insignificant below 400 nm, yet dramatic at longer wavelengths (Figure 3B). Excitation dependent emission (EWDE) was also reported for PEI-PCL PDOT and nitrogen doped carbon dots.[164, 193] Interestingly, the first material shows a linear red shift in the emission with the red shift in the excitation wavelength, but the later material shows a less dramatic shift in emission wavelengths with no sensitivity below 400 nm. Although no clear explanation exists for such difference or behavior, possibility of different surface defects was mentioned. Nonetheless, the EWDE indicates that what is observed is not a pure band-edge emission. Why PEI or the N-doped C-dots has a blue emission? They both have a strong absorbance at 360 nm and an emission at 460 nm with more or less similar EWDE. In case of the later, it was interpreted as π - π^* absorbance and surface-defect-related emission. Ji *et al* also reported EWDE from the nanowires formed with the gas phase deposition of pyromellitic diimiden with similar emission peak (360 nm excitation, 377 nm emission).[194] They suggest that this phenomenon may be due to the band gaps related to intermolecular potentials, size distribution of the wires and the separation between them, which prevents energy transfer between different wires. They did not exclude the possibility of solvent-nanowire interaction which may lead into broad range of localized singlet energies. One interesting point is that, such EWDE does not exist with nanowires where the separation between the imide units are larger [176] So, we do strongly believe that all of these emissions

are, at least partially, related to close proximity and aggregation of N's in N-doped or N-containing structures mentioned above.

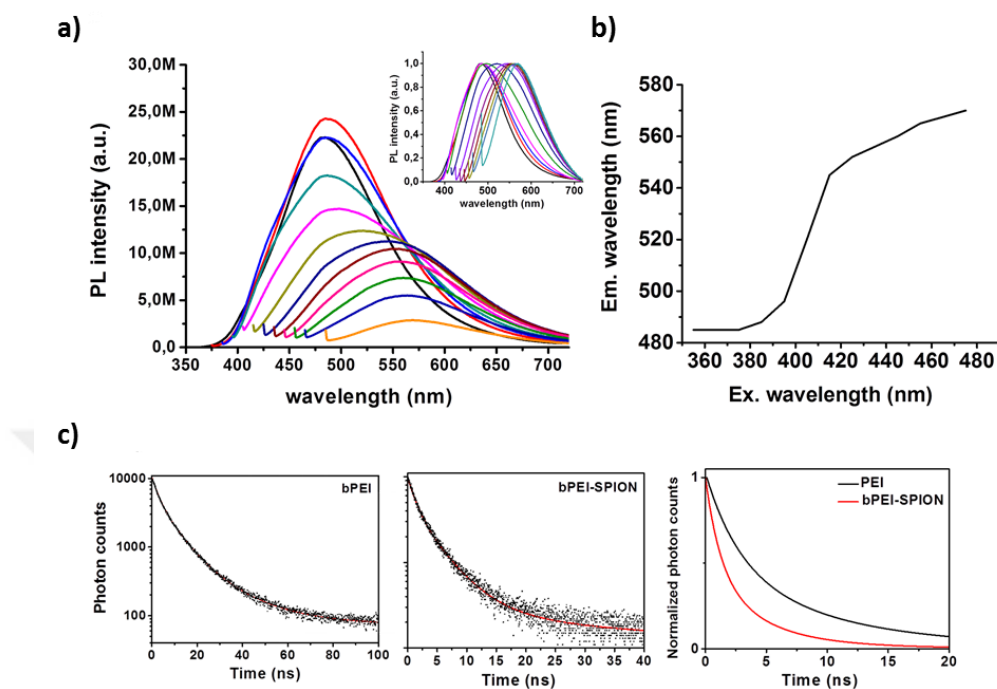


Figure 2. 6 a) PL spectra of bPEI-SPION excited at different wavelengths between 355-475 nm with 10 nm steps (Inset: excitation vs emission wavelength graph). b) Change in emission wavelength as a function of excitation wavelength. c) Fluorescence decay curve of bPEI-SPION (pH:5) at 485 nm, bPEI (pH:5) at 460 nm and normalized photon counts of bPEI and bPEI-SPION. pH adjustment was done with AcOH.

The average fluorescence lifetime (τ_{av}) of bPEI and bPEI-SPION are 8.23 and 14.15 ns, respectively with three different lifetime components (τ_1 , τ_2 and τ_3) in the range of 1.41-1.21, 4.23-4.17 and 12.8-21.81 ns (Table S1, Supporting Information). There is a nearly two fold increase in the slow component going from bPEI to bPEI/SPION (about 12.8 to 21.81 ns) but with reduced contribution to the average lifetime. The longest lifetime (τ_1) component of bPEI-SPION which is around 21.81 ns is far longer than the other reported values for quaternized, acidified or self-assembled structures [163, 164, 195] and, the luminescence intensity of bPEI-SPION is exceptionally higher

than the others, as well. This indicates that bPEI-SPION compsoition favor more radiative coupling of the exciton by reduce non-radiative paths and may have introduced new emissive centers which would support the observed EWDE, as well. Lifetimes reported for bPEI 10 kDa is 3.2 ns as is, 4.3 ns after oxidation and 6.2 ns at pH 2. [163] PDOT formed with 25kDa PEI reported to have a two component lifetime at 4.8 ns (56 %) and 1 ns (44 %). [164] In case of PAMAM dendrimers, two component lifetimes around 1.5 ns and 4-7 ns were reported which increased with increasing dendrimer generation and decreasing. [170] From a different aspect, Pastor-Peréz shows that rising molecular weight and methylation of amine groups also lead increase in the fluorescence life time of PEI. Although this increase can be attributed to the much dense delocalized electrons along the chain, but rigidity gained by the entaglement of polymer and thereby blokage of non radiative relaxations could be also the effective on the fluorensence life time. [163]

Table 2. 1 Fluorescence lifetime components of bPEI and bPEI-SPIONs

Sample	τ_1 (ns)	τ_2 (ns)	τ_3 (ns)	B_1 (%)	B_2 (%)	B_3 (%)	τ_{av} (ns)	χ^2
PEI	12.8 ± 0.76	4.23 ± 0.23	1.41 ± 0.14	20.39	58.56	21.05	8.23 ± 0.48	1.13
bPEI- SPION	21.81 ± 1.28	4.17 ± 0.15	1.21 ± 0.04	17.44	62.39	20.18	14.15 ± 0.89	1.02

2.4.3. Investigation of the origin of the luminescence from the bPEI-SPIONs

We have observed that it is also beyond simple surface adsorption which restricted the chain mobility or created N-rich clusters which causes such a dramatic enhancement in the luminescence intensity. In case of synthesis of bPEI-SPIONs by the room temperature

exchange of lauric acid (LA) with bPEI, such an enhanced emission or absorbance was not observed (Figure 2.7 a-b). Hence, absorbance and photoluminescence intensities do not depend on the pH characteristics in the solution given in the Figure 2.7 c-d.

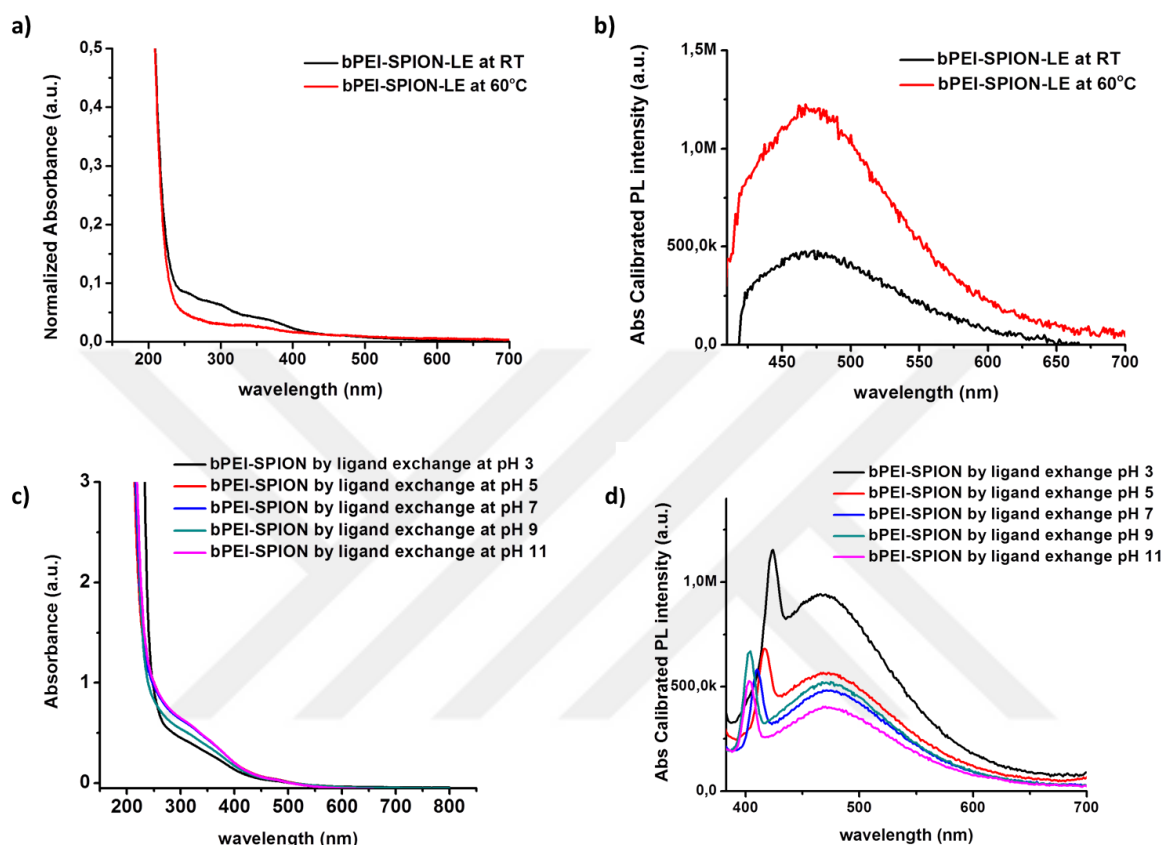
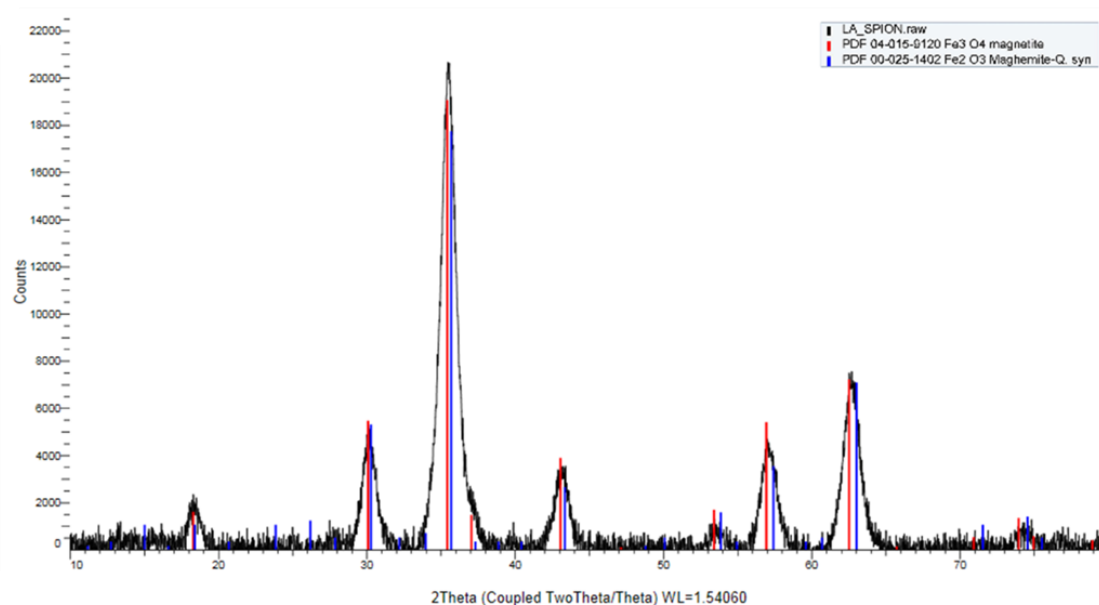


Figure 2. 7 a) Normalized absorbance and b) absorbance calibrated PL spectra of bPEI-SPION-LE prepared at room temperature (black) and at 60°C (red) c) normalized absorbance and d) absorbance calibrated photoluminescence spectra of bPEI-SPION prepared by ligand exchange method from lauric acid coated SPIONs at different pH values adjusted with acetic acid.

Crystal structure of both does not differentiated and the mixture of magnetite and maghemite. (Figure 2.8 a-b) Based on this, in order to understand the influence of reaction conditions on the exceptional luminescence of bPEI-SPION prepared by the in situ coating, couple of control experiments were performed: 1) bPEI was treated under the conditions mimicking the

in situ synthesis of bPEI-SPION without iron salts, but this was not sufficient to create the observed strong luminescence alone (Figure 2.9 a), **2)** In order to eliminate any negative effects that may originate from any lauric acid left on the SPION surface, bPEI was deposited on bare SPION prepared in the same way but without any organic coating, yet no remarkable enhancement in luminescence was detected (Figure 2.9 b) **3)** bPEI was treated only with the iron salts at room temperature and at 85 °C, separately, but again no comparable effect was observed (Figure 2.9 c).

a)



b)

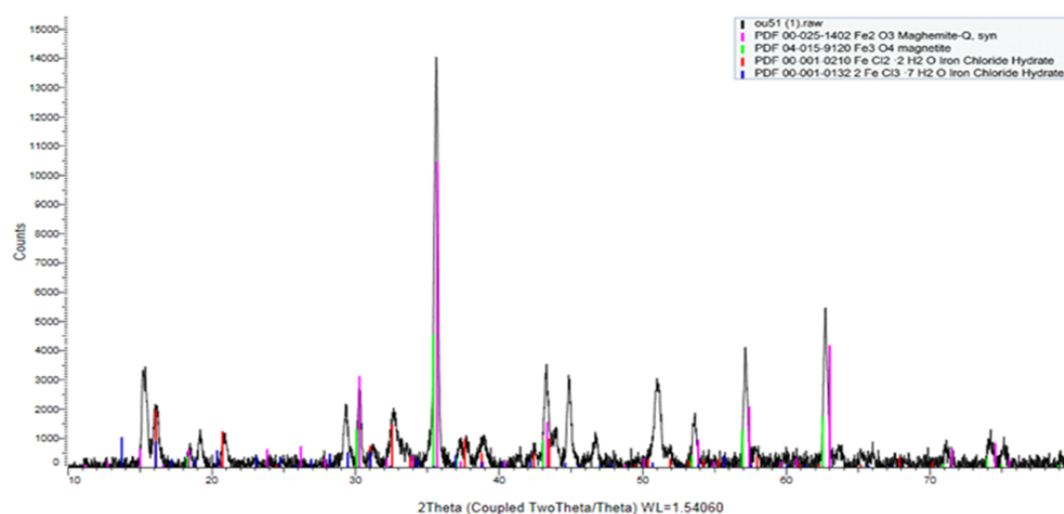


Figure 2. 8 X-Ray diffraction pattern of bPEI-SPIONs prepared (A) by ligand exchange method from lauric acid coated SPIONs and (B) with in situ coating method. Since presence of the polymer prevented the observation of diffraction pattern, it was burned under inert atmosphere. Extra diffraction peaks are probably originating from the impurities formed as a result of burning.

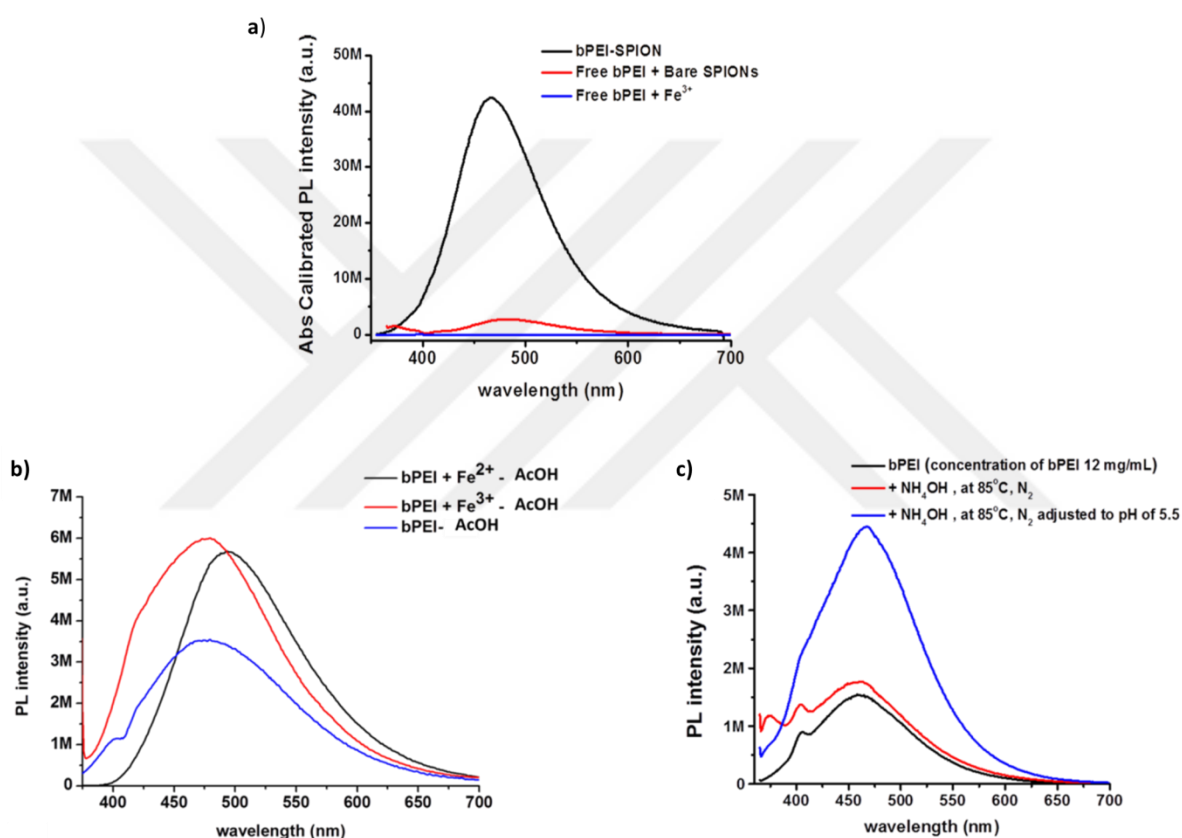


Figure 2. 9 a) PL spectra of *in situ* PEI coated SPION (black) , solution of free bPEI and bare iron oxide nanoparticles (red) and solution of free PEI/Fe³⁺ ions mixed at room temperature(blue). b) PL spectra of Fe²⁺ and Fe³⁺ doped bPEI solutions, stirred 30 mins at 85 °C under inert atmosphere and acidified with AcOH to pH 5 as compared to plain bPEI at pH 5. c) PL spectra of bPEI, bPEI treated with ammonium hydroxide at 85 °C for 30 min under inert atmosphere without acid treatment and after pH adjustment to pH 5.

Investigation of individual factors indicates that co-existence of bPEI and iron salts during the synthesis of the bPEI-SPION creates a special effect which produces such dramatic improvement in the luminescence intensity of bPEI. In couple of reports studying the blue luminescence of PAMAM [165, 167, 169] , linear and branched PEI [163], poly(amino ethers) [191] or polypropylene imine (PPI) [169] significant enhancement in emission intensity was gained after air exposure, ammonium persulfate (APS) or hydrogen peroxide (H_2O_2) treatments. This was generally attributed to the oxygen encapsulating capability of the interior part of these dendritic or highly branched structures and thereby, creation of fluorescent centers through oxidation of tertiary amines. Although weak, fluorescence obtained from a small tertiary amine, triethylamine (TEA), after oxygen doping supports such discussions that bases the origin of the luminescence to the presence of tertiary amines. [165, 163] Pan *et al* suggested that the formation of carbamate anions from the interaction of carbon dioxide with the amines of branched PEI ($M_n=600$ Da) and possibly n to π^* transition contribute to the blue luminescence of amine rich species.^[162] Some articles evaluated the phenomenon as formation of weak oxygen interaction rather than a strong covalent complex.[165, 169, 191] bPEI-SPIONs are prepared under Argon atmosphere so, we do not expect such event during the synthesis yet, when these nanoparticles were subjected to CO_2 bubbling for 5 minutes, a slight enhancement in the luminescence was detected. (Figure 2.10)

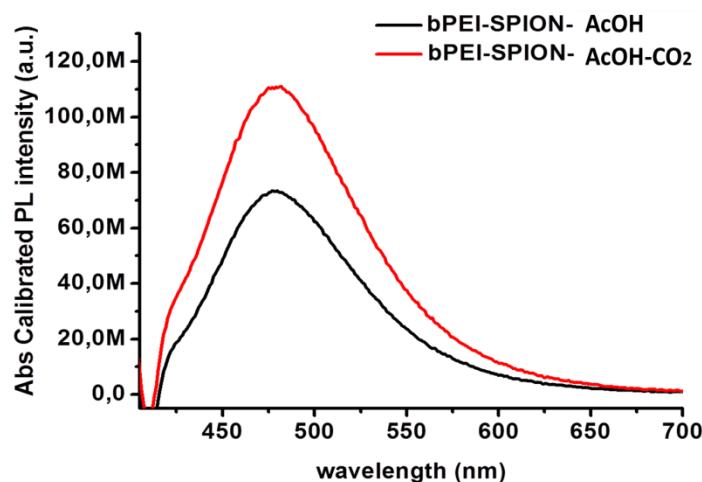


Figure 2. 10 PL spectra of bPEI-SPION treated with CO₂ and adjusted to pH 5.5 with AcOH. We have also noticed that bPEI shows a slight enhancement in the luminescence intensity upon aging with a significantly stronger absorption at 360 nm with no significant difference in the structure based on a FTIR analysis. (Figure 2.11)

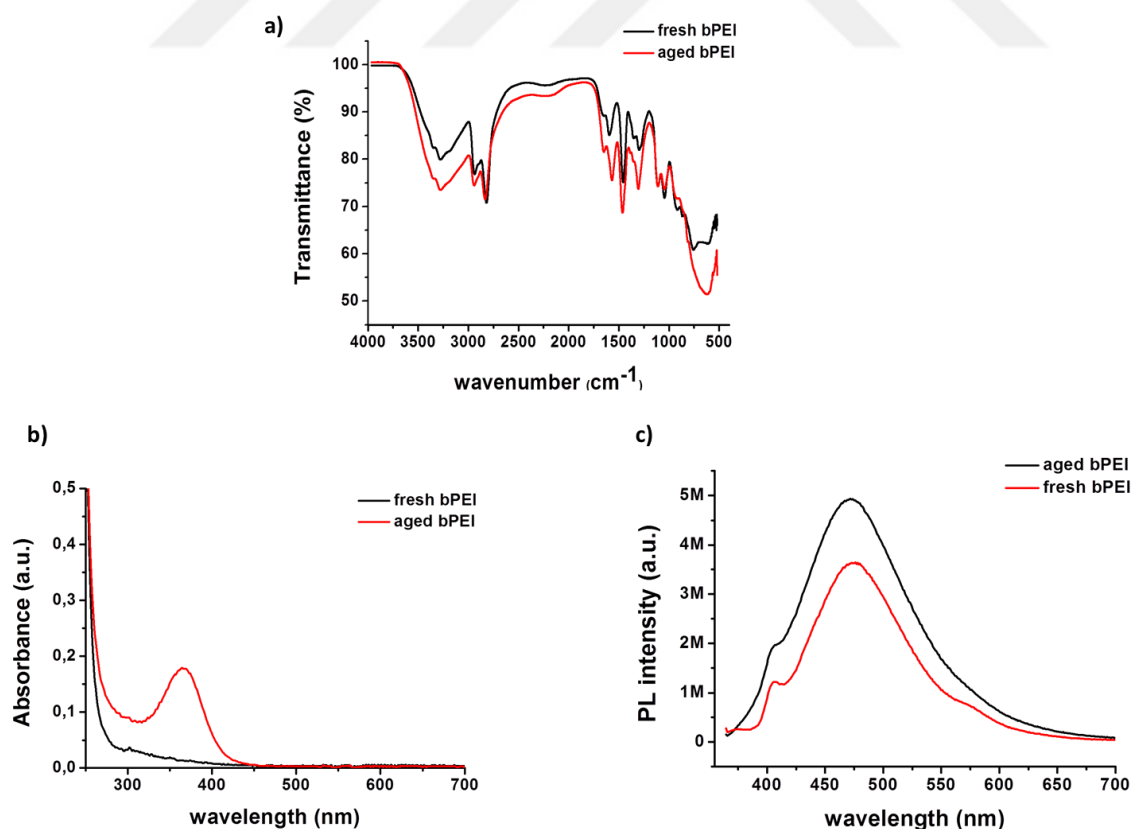


Figure 2. 11 a) FTIR spectrum of aged (with air) and fresh bPEI b) Absorbance and c) PL spectra of aged and fresh bPEI solution at the same concentration.

Due to the presence of SPIONs, $^1\text{H-NMR}$ spectroscopy was not a possible detection method for our samples. However, FTIR revealed some remarkable differences between the free PEI, bPEI-SPION prepared by ligand exchange (LE) versus in situ coating (Figure 2.12) : a 60 cm^{-1} shift to lower energy in C-N stretching band of in situ bPEI-SPION with respect to free bPEI and LE bPEI-SPION indicates a difference around nitrogen atoms. Additionally, lower percentage transmittance of N-H bending at 1546 cm^{-1} also confirms a change in the chemical environment during the in situ bPEI-SPION synthesis. Since such differences do not exist between fresh versus aged bPEI or in case of the nanoparticles prepared by the ligand exchange, it designates the origin of the bright blue fluorescence in bPEI is directly related with the change in the chemical environment of nitrogen atoms.

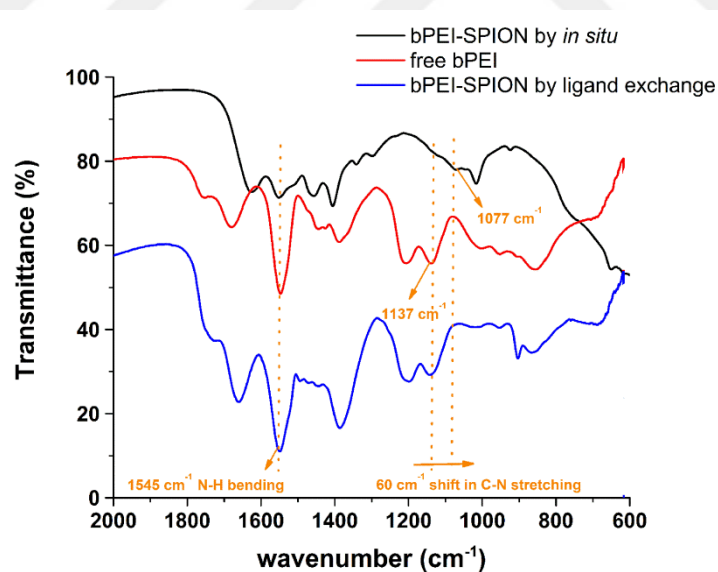


Figure 2. 12 FTIR spectra of free bPEI, and bPEI-SPION prepared by in situ coating of PEI (strong luminescence) and by ligand exchange (poor luminescence) of LA with PEI.

In order to understand the major chemical differences taking place at the iron oxide and bPEI components between the in situ coating, which produces the exceptional luminescence, versus

coating by ligand exchange, EPR and XPS experiments were performed on these two different nanoparticles and LA-coated SPIONs.

EPR is a useful, highly sensitive microscopic technique which gives detailed information about the spin states of the Fe and its environment. [196-201],[202-204] Electronic transitions measured with EPR are sensitive to distortions in the crystal field. Thus g-factors of ions which have unpaired electrons, such as paramagnetic Fe^{3+} , depend on the substitution and site incorporation of Fe^{3+} , hence EPR spectroscopy can distinguish the electronic neighbourhood of Fe^{3+} ions. Free Fe^{3+} ions give EPR resonance at $g=2.0$. [205] However, depending on the distortion of their crystal field, the g-values can be deviated from 2 to 9 [206]. If paramagnetic centers partly consist of superparamagnetic states like Fe^{3+} in case of Fe_3O_4 crystal, strong spin-spin exchange coupling occurs between those Fe^{3+} ions which drastically broadens the EPR linewidth. Superparamagnetic states also give broad EPR peaks centered around $g \sim 2$ [207]. All three samples have active iron EPR centers above the detection limit (10^{10} spins [208]) of EPR spectroscopy (Figure 2.13)

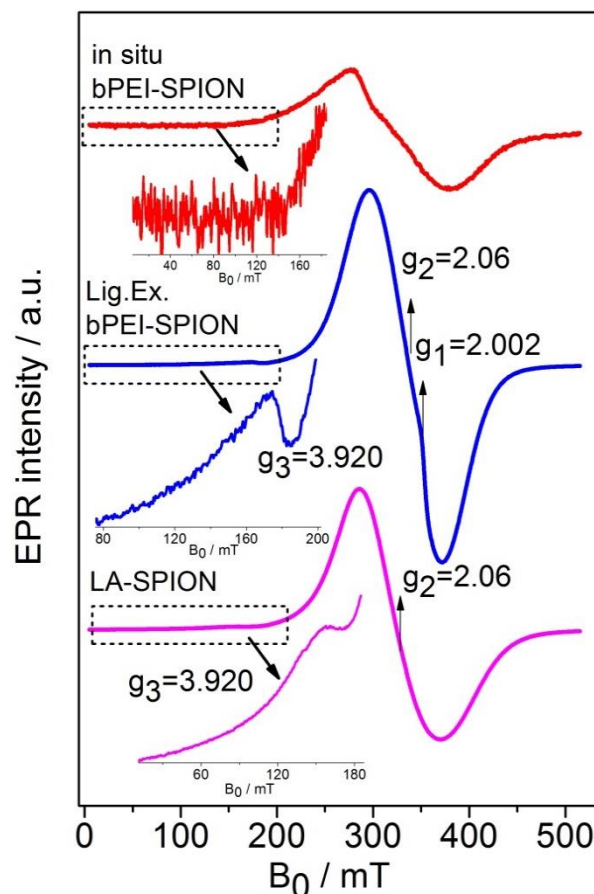


Figure 2. 13 Room temperature X-Band EPR spectra of bPEI-SPION synthesized in the presence of bPEI (in situ), by ligand exchange of LA with bPEI from the LA- SPION and LA- SPION.

In order to extract the exact corresponding g-factors of each overlapped Fe center, it is necessary to analyze the data in integrated form with curve fitting (Figure 2.14). From the deconvoluted spectra, g factor of each overlapped signal can be determined accurately.

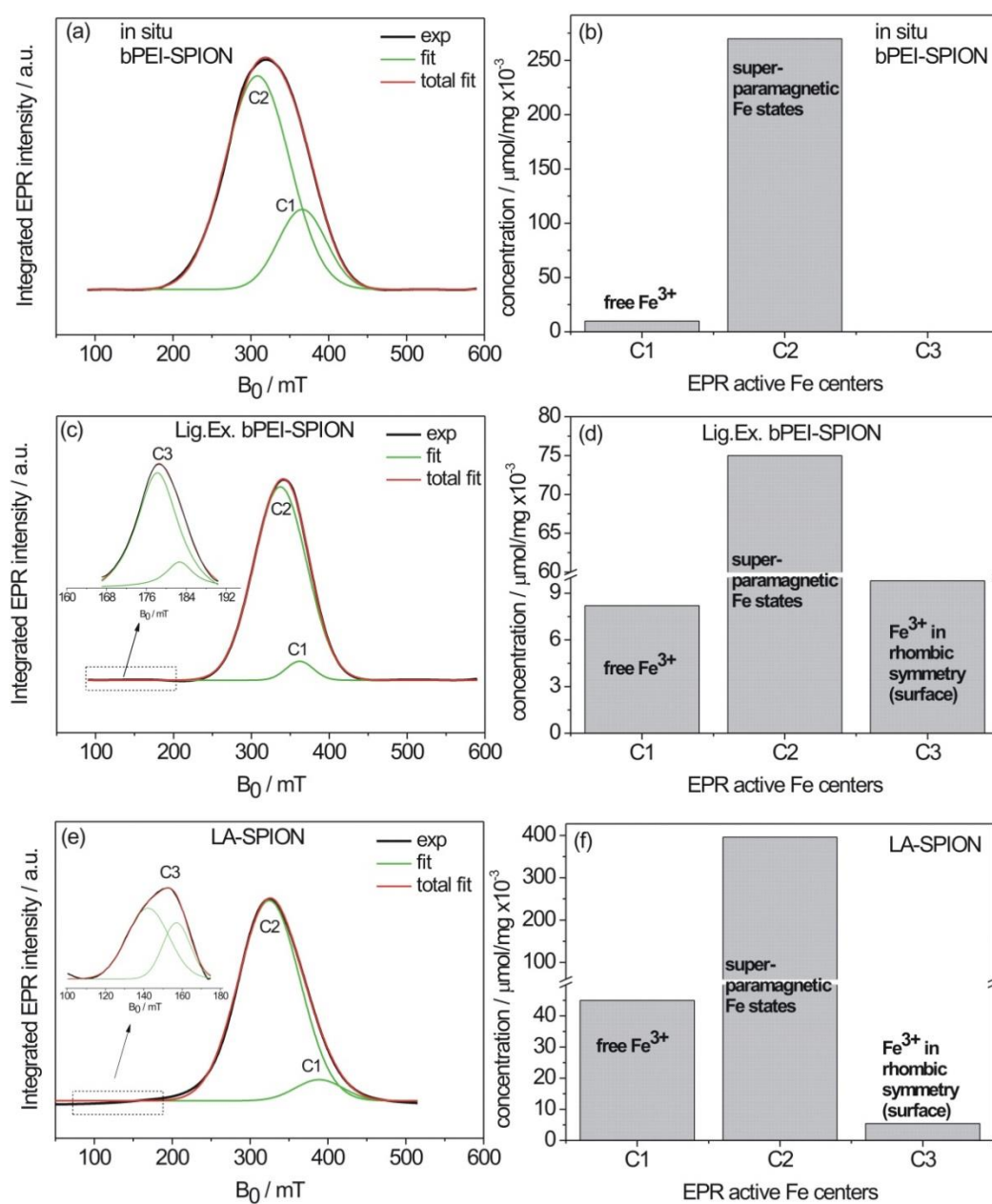


Figure 2. 14 (a, c, e) The absorption signal of each Fe center. EPR line can be deconvoluted in three different gaussian peaks (insets) and (b, d, f) the absolute concentration of each Fe center.

The EPR spectra of the LE bPEI-SPION is very similar to LA-SPION with three nonequivalent magnetic Fe centers and very different than the strongly luminescent in situ bPEI-SPION which has two distinct nonequivalent magnetic Fe centers. This suggests that

the key parameter is the coexistence of iron salts and PEI simultaneously during the synthesis of SPION. The Fe center responsible from the half-field signal at the $g_2 = 2.060$ represents the superparamagnetic state where magnetic dipoles are randomly oriented.[202] It is also known that all samples of surfactant-coated SPION nanoparticles show very small macroscopic magnetization. The signal at $g_1 = 2.002$ is very close to free electron value ($g_e = 2.0023$) therefore, related to the delocalized electrons of free Fe^{3+} ions which are not associated with the crystal. [197] The lower magnetic field signal at $g_3 = 3.920$ is a typical EPR signal for rhombohedrally distorted, magnetically decoupled high spin Fe^{3+} ions [202], meaning that the environment of Fe^{3+} ions are strongly deviated from octahedral to trigonal symmetry. [209] This type of Fe^{3+} ions does not exist in the in situ bPEI-SPION. Decoupling mostly occurs from the core, thus most probably these ions are on the crystal surface of LA-SPION and LE PEI-SPION which vanished when SPION was produced in the presence of bPEI. Overall, EPR data strongly indicates that LA coated SPIONs and its ligand exchanged form with bPEI has similar composition and is quite different than in situ bPEI-SPION. In addition, concentration of each paramagnetically active Fe states was quantified by spin counting and the absolute values were given in Table 2.2 [210]

Table 2. 2 Fe^{3+} concentration of iron oxide nanoparticles obtained from both EPR and ICP-MS.

	In situ bPEI-SPION	Ligand exchange bPEI-SPION	LA-SPION
Total Fe in samples measured by ICP ($\mu\text{mol/mg}$)	0.16	0.14	2.7
Free Fe^{3+} ($\mu\text{mol/mg}$)	0.0099	0.0082	0.045
Superparamagnetic Fe-states	0.0270	0.075	0.396

($\mu\text{mol}/\text{mg}$)			
Surface Fe³⁺ ($\mu\text{mol}/\text{mg}$)	0	0.0098	0.0054
Total Fe³⁺ ($\mu\text{mol}/\text{mg}$)	0.0099	0.018	0.0504

Based on this analysis, all Fe concentrations calculated from EPR signals are lower per mg in LE bPEI-SPION than LA-SPION. This is in agreement with the ICP data and has two main reasons: 1. there is dramatically more organic content in the former than later as confirmed by the ICP; 2. some of the particles could not be recovered during the ligand exchange. Replacing LA coating with bPEI by ligand exchange did not change the ratio of superparamagnetic/free Fe³⁺ ratio, but dramatically decreased the superparamagnetic/surface Fe³⁺ indicating better capture of the surface. Yet, there are three very important differences between the strongly luminescent in situ bPEI-SPION and the other two: 1. in situ coating approach completely eliminated this rhombic surface Fe³⁺; 2. superparamagnetic/free Fe³⁺ ratio decreased dramatically in the case of in situ bPEI-SPION; 3. Assuming that these nanoparticles are composed of only Fe³⁺ and Fe²⁺, in almost same amount of samples (0.8 mg Fe/mg sample in luminescent and 0.7 mg Fe/g in LE bPEI-SPION, as determined by ICP), there is much less Fe³⁺ in the luminescent ones, indicating that a significant amount of Fe³⁺ got reduced to Fe²⁺ in the in situ synthesis. Based on the absence of relevant signal in the EPR spectrum, reduced irons are the rhombohedrally distorted surface Fe³⁺ which is in interaction with bPEI. If so, there should be an oxidation in the bPEI. As discussed above, several reports indicate the importance of amine oxidation in the enhancement of the weak blue luminescence of PEI. Detection of such oxidation is quite difficult since NMR is not possible and FTIR provided some indications, but not a clear support.

A significant difference in the N1s core level binding energies can be detected in the XPS (Figure 2.15) supporting an oxidative event. N1s peak of the LE bPEI-SPION, can be deconvoluted in three peaks at 398.44 eV (2%), 399.7 eV (80%) and 400.6 eV (18%). Highest binding energy peak is usually attributed to the H-bonded or protonated amine. N1s peak of the strongly luminescent *in situ* bPEI-SPION, can be also deconvoluted in three peaks at 398.44 eV (2.5%), 399.8 eV (61.5%) and 401.8 eV (36%). The decrease in the amount of the major peak at 399.8 eV and the shift of the 400.6 eV peak to higher binding energy along with an increase in its content supports oxidation of amines. Since the LE bPEI-SPION was prepared from already made iron oxide, no amine oxidation and Fe^{3+} reduction are expected during the ligand exchange, hence no significantly enhanced luminescence.

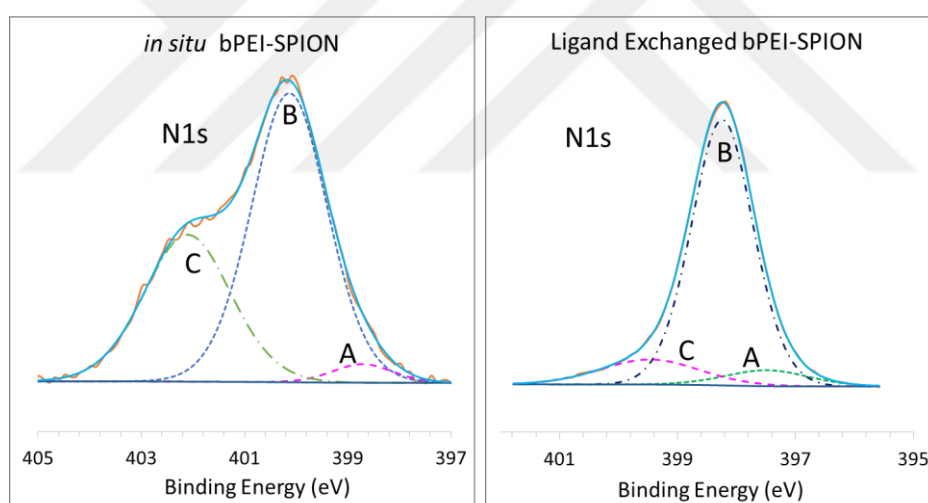


Figure 2. 15 XPS N 1s core level of strongly luminescent *in situ* bPEI-SPION (left) and ligand exchange bPEI-SPION (right).

Based on these, we suggest that adsorption of bPEI on SPION surface enhances the blue luminescence of bPEI, acidification improves it more strongly, aging or air oxidation has a significant positive effect as well in the enhancement of the blue emission, yet, the major contribution to the exceptionally strong luminescence intensity of bPEI-SPION prepared by

the in situ coating of iron oxide with bPEI is the oxidation of PEI coupled with all these factors.

2.4.4. Density Functional Theory Calculations

In order to model the iron oxide-PEI interactions in a simple form, we studied the electronic and structural changes of PEI upon doping with Fe^{+2} and Fe^{+3} ions by Density-Functional-Theory (DFT) calculations. But first, structures of a large set of PEI oligomers (which include various combinations of primary, secondary and tertiary amine sites) are optimized by B3LYP functional with 6-31g(d,p) basis set using Gaussian 09 software. There are several trends observed in these neutral PEI oligomers. First of all, upon comparing the ground state energies of oligomers containing the same number of N atoms, those with the larger numbers of secondary sites are more stable. This is most likely due to the structural flexibility of the linear oligomers. On the other hand, HOMO-LUMO gaps seem to be independent of the size and the branching. This may also support the literature data indicating possibility of only blue luminescence from PEI at all molecular weights, branched or linear. But more importantly it allows us to study the interaction with oligomers, here. Then, the protonation of PEI was studied and average stabilization energies are calculated as -235, -243 and -248 kcal/mol, respectively, for primary, secondary and tertiary sites, which is actually following the order of basicity. Electronic spectra of both neutral and protonated PEI oligomers are calculated again with DFT, this time with M062x/6-31g(d,p) set. The lowest absorptions are all around 170 nm for neutral oligomers. If the primary amines are protonated, then the lowest absorptions shift to 240 nm. For protonated secondary and tertiary amines, these numbers are 200 and 180 nm, respectively.

Lastly, the geometries of Fe^{+2} and Fe^{+3} doped PEI oligomers are optimized with M062X/6-31g(d,p) set. The wavefunctions usually display UHF-RHF instability, so all calculations are

carried out first by stabilizing the wavefunctions. The spin multiplicities of Fe^{+2} and Fe^{+3} are 5 and 6, respectively, and the same multiplicities are found to have the lowest energies for Fe^{+2} and Fe^{+3} doped PEI. Similar to protonation, Fe^{+2} and Fe^{+3} can attack a number of sites on PEI oligomers. However, computed geometric and spectroscopic changes are more radical with the ion doping. The major structural changes occur if the iron atom can twist PEI to form cage-like structures (Figure 2.16)

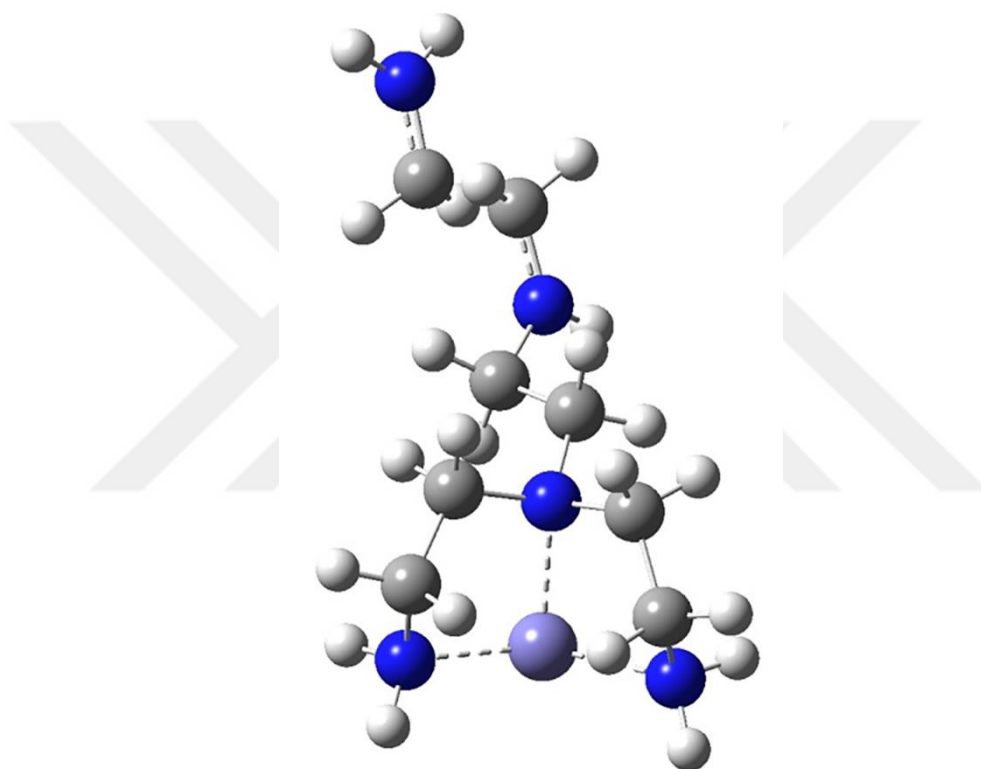


Figure 2. 16 An example of a cage-like PEI- Fe^{2+} complex.

Upon doping with a Fe^{+2} ion, if the structure of PEI does not change much, then the stabilization energies are around 185 kcal/mol which is not much different than the protonation. However, stabilization of the cage-like complexes are 300 kcal/mol or more. In all cases N-Fe distance is around 2.07-2.13 Å. The changes of the electronic properties of Fe^{+2} doped PEI are summarized in Table 2.3.

Table 2. 3 The changes of the electronic properties of Fe^{+2} doped PEI.

Site	Mulliken Charge on Fe	Number of Parallel spins on Fe	Lowest Absorption (nm)
Primary	0.75	3.0	410
Secondary	0.70	3.0	770
3-membered cage	1.20	3.9	510

For the cases where a Fe^{+2} is attached to a primary or secondary amine without distorting the structure of the oligomer, there is an electron transfer from Fe^{+2} to PEI remembering that Fe^{+2} ion in the gas phase should have $q=2$ and $S=4$ and the transferred electron is coupled with one of the d electrons of iron and reducing S to 3. But in the cage-like structures, iron ion seems to retain all of its d electrons. In all cases, the reasonably strong absorptions in the visible range are observed.

The situation with Fe^{+3} shows larger variations due to the larger charge on the ion. In general stabilization energies are larger than those of Fe^{+2} complexes and they reach 700 kcal/mol for cage-like structures. Fe-N distances are slightly longer. The electron-transfer from ferric ion to PEI again occurs in various degrees depending on the structures formed. The gas phase Fe^{+3} ion should have $q=3$ and $S=5$. In the studied complexes, the total spin on Fe is always slightly less than 4 showing the loss of an electron from PEI side, even for the cage-like structures. The Mulliken charges are found to be as low as 1.0 again pointing out possible oxidation of PEI. Fe^{+3} doped PEI oligomers show absorptions within the 300-600 nm range.

2.4.5. Evaluation of Luminescent bPEI-SPION for In Vitro Gene Delivery and Imaging

Gene transfection

Usually the main purpose of using PEI coating on SPIONs is to provide a cationic nanoparticle suitable for gene binding and transfection. As a model cargo, a dsDNA, poly I:C (PIC), was used. Due to its labile structure and sensitivity to enzymatic degradations, PIC needs an efficient carrier to pass through the cell membrane. Polyanionic PIC was loaded to bPEI-SPION electrostatically. Complete binding of PIC was achieved at bPEI-SPION/PIC weight ratio of 2/1. PIC loading onto bPEI-SPION did not change the luminescence intensity or the peak position of bPEI-SPION which is highly desirable (Figure 2.17 a-b).

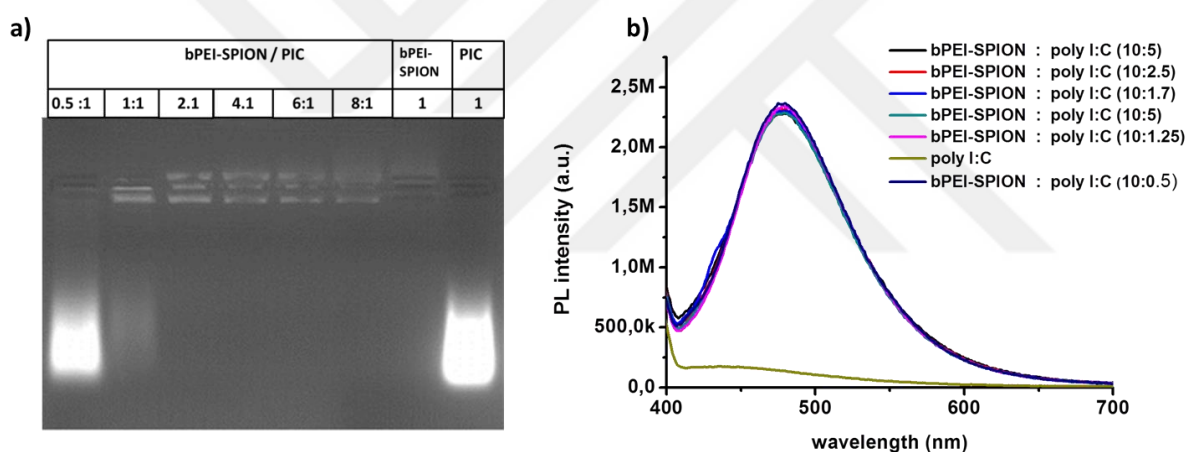


Figure 2. 17 Determination of the PIC binding capability of bPEI-SPIONs and change in their fluorescence properties after binding with PIC. a) Gel electrophoresis showing the binding efficiency of PIC to bPEI-SPION (1.2% agarose gel, HBG buffer, pH:7.4) b) PL spectra of poly I:C bounded bPEI-SPIONs at different ratios (w/w).

In order to determine the cytocompatibility of bPEI-SPIONs itself and evaluate its transfection efficiency, viability of HeLA cells treated with free bPEI (0.4 to 16 $\mu\text{g/mL}$), bPEI-SPION and bPEI-SPION/PIC was determined with the MTT assay (Figure 2.18). For the sake of healthy comparison, dose of the nanoparticles were determined based on their

bPEI content measured by TGA. In case of free bPEI, significant dose dependent toxicity was recorded between 5 to 16 $\mu\text{g/mL}$. But, when same amounts of bPEI is bound to SPION, no significant toxicity was observed, until 24 $\mu\text{g/mL}$ nanoparticle concentration which consists of 8 μg of SPION and 16 μg bPEI/mL. At this highest dose bPEI-SPION cause 41% but free polymer causes about 97 % cell death. At the lower doses of 16 and 8 $\mu\text{g/mL}$ which contains 10 and 5 $\mu\text{g/mL}$ bPEI no cell death was observed. This is a dramatic enhancement in cytocompatibility which may be partially due decreased overall positive charge upon adsorption of some amine groups to crystal surface [211] and may be partially due to oxidation of amine groups. [212] Cytocompatibility of the delivery vehicles are very important.

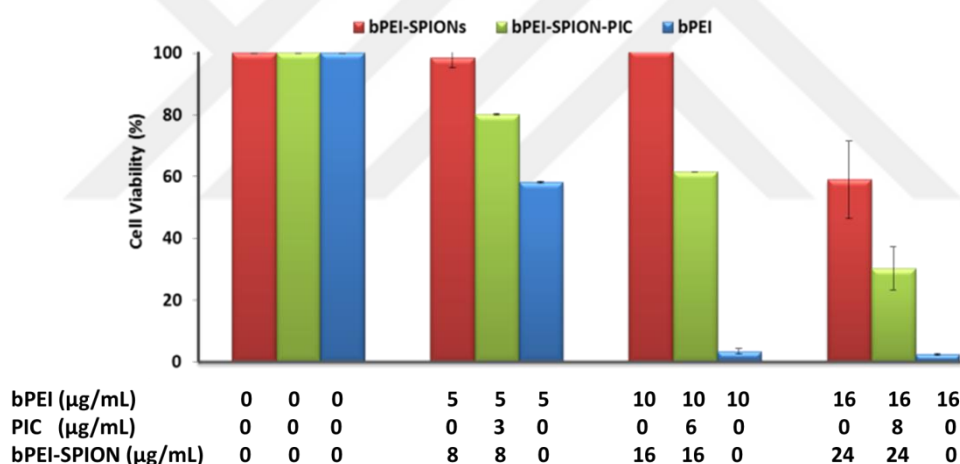


Figure 2. 18 Viability of HeLA cells treated with free bPEI (blue), bPEI-SPION (purple) and bPEI-SPION-poly I:C (green) for 48h determined by MTT assay. Free bPEI 25 kDa, PIC and bPEI-SPION concentrations identifies the content of each component in the formulation given to cells in each column.

Successful delivery of PIC to HeLa cells and its release from the nanoparticle should cause significant cell death. Hence, viability decreased by 20-40-70% with 3-6-8 μg PIC delivered

with increasing dose of the bPEI-SPIONs/PIC. Especially at 8 and 16 μg nanoparticle dose, all the toxicity is originating from the PIC delivered into the cells, which is excellent.

Imaging

One of the most attractive feature of this bPEI-SPIONs is the strong blue emission which makes extra fluorescent tagging unnecessary. As can be seen in the fluorescence microscopy images of the cells treated with bPEI-SPION, even at low concentrations (10 μg nanoparticle/mL), a strong optical signal was detected in the cytoplasm of the cells, proving these particles as good optical imaging agents. PIC loaded nanoparticles also displayed a similar strong blue luminescence in the cytoplasm of cells, allowing one to track the destiny of the delivery vehicle optically. On the other hand, free bPEI shows a very weak signal (Figure 2.19). Most probably this is why it has not been recognized in fluorescence microscopy studies before.

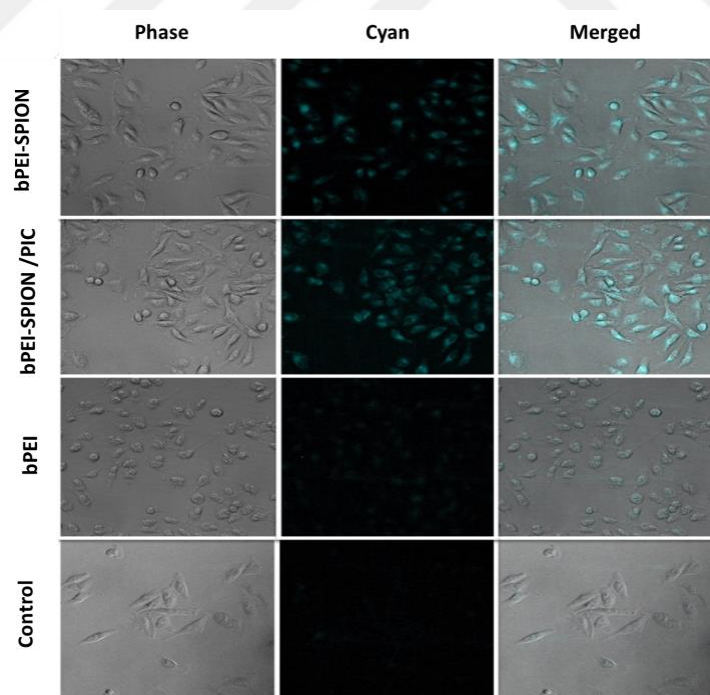


Figure 2. 19 Fluorescence microscopy images of free bPEI, bPEI-SPION and bPEI-SPION-PIC (at 10 μg bPEI/mL dose) treated HeLa cells at 20X magnification (λ_{ex} = 358, λ_{em} =480 nm)

Since SPIONs are most valuable in MRI, magnetic properties of bPEI-SPION was studied with vibration sample magnetometer (VSM) at room temperature (Figure 20 a). Particles show superparamagnetic character with remanence and coercivity values near to zero. In order to investigate MRI potential of bPEI-SPIONs, it was imaged at the same concentration used for fluorescence imaging (10 $\mu\text{g/mL}$ bPEI which is 16 $\mu\text{g/mL}$ nanoparticle) in 3 Tesla clinic MRI with DI water as a reference. It shows a significant dark image compared to DIW even at this dilute conditions (Figure 20 b). This image, along with the strong intracellular blue luminescence (Figure 19) makes these particles great candidates for dual imaging: optical and magnetic.

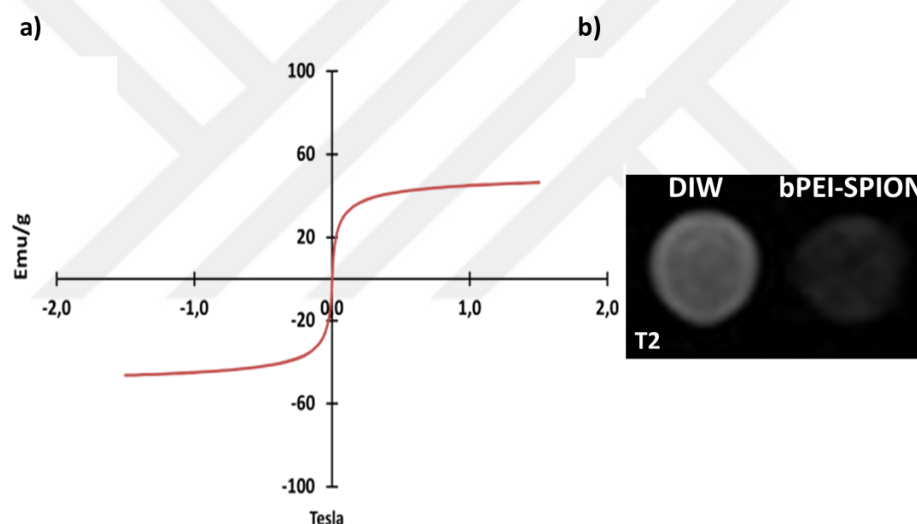


Figure 2. 20 Magnetic properties of bPEI-SPIONs. (A) Magnetization loop (ambient conditions, 0.5 emu/g magnetic field) (B) T2 weighted image of deionized water (DIW, SI: 163) and bPEI-SPION (iron content = 0.9 $\mu\text{g/mL}$, SI: 17) at room temperature using 3T MRI scanner.

2.5. Conclusions

In this study, a tremendous enhancement in the poor, mostly unrecognized and unutilized blue fluorescence of bPEI, was investigated when it is *in situ* deposited on SPION crystals. This

is an exceptional finding especially since SPION has very strong absorbance in the visible region. Luminescence of bPEI-SPION is about 1200 times stronger than free bPEI at the same polymer content in most favourable acidic conditions and once achieved is independent of further pH adjustments. Here, we have tried to identify the factors creating such an enhancement in the luminescence of bPEI and the origin of the luminescence. Firstly, it is clearly demonstrated that it is not just the surface adsorption and hence increased rigidity or close proximity of the amine groups but yet confirmed the strong influence of acidification on the enhancement of luminescence intensity. Then, it was also shown that it is not the iron salts, ammonium hydroxide, heat treatment or air oxidation which can cause the observed strong luminescence, indicating that the whole recipe (in situ coating method) used in the synthesis of bPEI-SPION is necessary for such effect. XPS and EPR studies indicated the PEI oxidation and Fe^{3+} reduction during the *in-situ* coating method. Tendency of PEI to reduce iron salts is also suggested by DFT calculations performed. In addition, based on our calculations, HOMO-LUMO gap is relatively insensitive to the molecular weight and the branching of PEI, which in part may be the reason behind observing only blue luminescence with any kind of PEI, PAMAM, N-doped C-dots or diimid systems. This has to be studied further. Lastly, EWDE and three component luminescence decay indicates existence of different emissive centres and/or environments with longer average lifetime and significantly longer slower component of bPEI-SPION compared to bPEI.

Overall, combination of amine oxidation, surface adsorption of bPEI coupled with final acidification is the major affecters behind such strong blue emission observed from bPEI-SPION. Of course, restricted mobility, close proximity of amines, distribution of such factors within the same particles as well as reduction in the free lone pair

electrons of amines and exciton formation can be the origin of the luminescence, which may be understood better if all similar systems are studied together, in the future studies.

Besides, these self-luminescent iron oxide nanoparticles are superparamagnetic and produce remarkable T2 signal as well at the concentration of their strongly fluorescent state which addresses the magnetization of iron oxide core is preserved. Oxidation of amines on bPEI does not significantly affect its cationic nature, in other words its gene loading capability. Functionality of oxidized bPEI on SPIONs was confirmed with the efficient loading and delivery results of PIC. When PIC was loaded to these nanoparticles (37 weight %), it was effectively delivered to HeLa cervical cancer cells and killed about 50 % of the cells in 48h. Hence, bPEI-SPIONs are highly cyto-compatible although they are cationic and produces bright blue luminescence inside the intracellular environment, as well.

Overall, discovery of the amine oxidation as one of the key factors enhancing the luminescence of PEI contribute significantly to the literature of luminescent polyamines. From the practical perspective, this invention now points out optical imaging modality for PEI-SPIONs which brings out a tremendous advantage in development of SPIONs for dual mode imaging. Finally improved biocompatibility and therapeutic oligonucleotide delivery renders these particles invaluable as multifunctional theranostic compositions that can be developed in a single step.

CHAPTER 3

3. SYNTHESIS AND CHARACTERIZATION OF MULTIFUNCTIONAL PROTEIN COATED SPIONs FOR TARGETED miRNA DELIVERY FOR CANCER THERAPY

3.1. Introduction

Recently, it was found that microosomal RNAs (miRNAs), small non-coded strands of RNA, show significant effects on cancer cell progress by regulating oncogenes and tumor suppressor genes.[213-215] miRNAs target different gene expressions and regulate cell signaling therefore mis-expression of miRNAs may participate in cancer formation.[216, 217] Since miRNAs have a crucial role in modulating gene expression, they have a high potential as a therapeutic tool (miRNA mimic) for cancer therapy besides being a therapeutic target (miRNA antagonist).[218-221] Antisense therapy using specific miRNA families are desired to target specific oncogenes such as bcl-2, c-myc and ras family at the translation stage of the protein synthesis.[222-224] For example, expression of bcl-2 was significantly reduced *in vitro* with G3139 oncogenes delivered in lyposomal carriers.[223] Successful inhibition of cell growth in melanoma and adenocarcinoma were also reported when c-myc gene was targeted to these cells with antisense therapy.[222] As an alternative approach, developing antisense targeting of ribozyme provided successful *in vitro* and *in vivo* treatment of colon cancer.[224] miRNAs as antisense oligonucleotides provide more successful treatment of cancer by synergistically regulating a broad range of genes compared to conventional antibody or small ligand therapies.[225, 226] Besides cancer cells, miRNAs target even tumor promoting endothelial cells or tumor associated fibroblasts and show reduced immune

response and low cytotoxicity compared to plasmid DNAs or ligand/ antibody based drugs.[227, 228] Therefore, miRNAs are highly beneficial and practical tools for cancer therapy. However, due to their small size and low molecular weight compared to plasmid DNAs and siRNAs, miRNAs need advance delivery systems in order to overcome several biological and physical barriers. Therefore, various local or systemic miRNA delivery strategies were developed to improve their penetration to the desired tissue before renal clearance and endosomal degradation. Especially systemic miRNA delivery has been intensely studied with viral and non-viral delivery approaches. Although viral entities show efficient transfection of miRNAs, they are immunogenic and mutagenic.[229, 230] Therefore, relatively non-toxic non-viral delivery agents are stronger candidates for practical miRNA delivery. Wide range of biocompatible formulations such as lipid derivatives [231], polyethylene imine (PEI) [232], cationic dendrimers [233], poly (lactide-co-glycolide) (PLGA) particles [234], collagen derivatives [235], silica nanoparticles [236], iron oxide nanoparticles (SPIONs) [237], gold nanoparticles (Au NPs) [238] are under consideration for both *in vitro* and *in vivo* anticancer therapy studies. Biocompatibility, high gene loading capacity, preservation of miRNA during blood circulation, sustained release at the target, potential for co-delivery with another therapeutic agent, high ratio of surface moieties to volume for functionalization, targetability and small sizes are major requirements for a good miRNA delivery vehicle. However, despite of the advantages of existing delivery systems, miRNA delivery still suffers from toxicity, non-specific uptake and immune attack which are directly caused by the positive charges on delivery agents [239, 240, 231] Therefore, the most critical issue is the toxicity originating from these cationic compartment which alternative new materials. From this aspect, looking into nature`s ways of gene silencing and intercellular miRNA circulation may provide inspiring ideas for more elegant miRNA carriers.

The regulation of gene expression by miRNA has been shown by RNA induced silencing complex (RISC) composing of primarily nearly a 22 nucleotide single strand RNA and a ribonucleoprotein, Argonaute (AGO). [241, 239, 240, 242] In RISC, the RNA strand basically guides AGO to target mRNA using the base complementarity. If guide and target strands are complementary with each other, catalytically active site of AGO slices the mRNA to inhibit translation.[243, 244] It was also observed that mature miRNAs exist in nuclease containing blood plasma and serum without any degradation[241, 245, 246] and mediates cell-cell communication.[231] Some groups suggest that, AGO2 protein is also responsible from the circulation and protection of majority of these extracellular miRNAs in biological media.[247, 244] The idea of “a natural miRNA carrier” is an inspiring concept for the development of highly needed next generation miRNA and further for gene delivery systems. Hence, based on the miRNA carrying capability of AGO2 in extracellular media, incorporation of AGO2 protein on a nanoparticle is proposed for the development of miRNA delivery which may eliminate or dramatically reduce the toxic effect of poly-cations which are conventional gene carriers.

SPIONs are excellent candidates for AGO2 conjugated nanoparticle delivery systems since they are biocompatible [245], magnetically controllable [248, 249], MRI visible [250] and available for surface functionalization [251]. SPION based transfection agents are preferred in the literature and many examples demonstrate the use of its multifunctional theranostic nature: MRI guided and magnetically assisted gene delivery [252, 248, 253], targeting with the antibody or small ligands [252, 254], multimodal imaging with fluorescent moieties [255] are various beneficial approaches introduced with the SPION based gene therapy systems. Smart miRNA delivery strategies with SPIONs for are also reported in the literature : Schade *et al* developed a magnetic polyplex to deliver mir-335 to human mesenchymal stem cells in order to improve their regenerative effect.[237] They basically condensate miR-335 with PEI

and conjugated to SPION surface with biotin-streptavidin recognition and observed a sustained release of miRNA and efficient genetic modification of stem cells. Sun *et al* demonstrated a miR16 carrier construct using PEI-PEG SPIONs to exclude drug resistance of SGC7901 to adriamycin and reported an efficient decrease in tumor volume when combined adriamycin with SPIONs/miRNA complex.[256] Moreover, they imaged the tumor tissue with dual modality: MRI and fluorescence. Leder *et al* aimed to deplete the level of target miRNA Let7g which are regulators of cell proliferation in hepatocytes and achieved this by using covalently antisense locked nucleic acid-silica based SPION conjugates which can be tracked by MRI and fluorescence imaging.[257] Interestingly, Lee *et al* produced self-assembling magnetic signal-on iron oxide based nanoparticle system to probe intracellular miRNA during neurogenesis [104]. They based the sensing mechanism on construction of 5' and 3' adaptor oligonucleotides conjugated to nanoparticles and their hybridization with miR linker. Resulting aggregates were dispersed in the presence of miR124a which can be monitored by the quenched T₂ signal in MRI.

Targeted anticancer therapy is the most beneficial way to eliminate undesired interactions of therapeutics with the healthy tissue and improve the success of the therapy.[258-261] Various strategies exist for the molecular targeting of cancer. Since most cancer types overexpress diverse types of receptors on cell surface for fast growth and proliferation, these overexpressed receptors are the most popular targets in the cancer research. Human epidermal growth factor receptor 2 (Her2) regulates cell signaling which promotes cell proliferation or tumorogenesis. Therefore, its expression is amplified in many different cancer types. Especially it is an important indication of breast cancer since it is overexpressed in 15-30 % of breast cancers. [262] Targeting strategy is successfully applied to treat Her2 overexpressed breast cancer [263, 264] and various nanoparticles coupled with antiHer2 are much selectively

internalized by the breast cancer cells through receptor mediated endocytosis.[265, 266, 108, 267]

Here, we propose small SPION-AGO2 based novel composition as a next generation miRNA delivery system and for the demonstration of idea targeted miR376b delivery to HER2 overexpressed SKBR3 and MDA-MB-453 breast cancer cells to regulate autophagy related protein expression is suggested. For this purpose, small hydrodynamic size, colloidal stability, sufficient AGO2 conjugation for miRNA binding, sufficient antiHer2 labeling for effective targeting and also conjugation of fluorescent dye for optical detection are suggested. This chapter will provide the details of different design and conjugation strategies and the optimized protocol to achieve such particles.

Since AGO2 is an expensive component, instead of direct synthesis of SPION with AGO2 coating which would require a large amount of protein, surface functional SPION is planned as the core material to which AGO2 and antiHer2 can be conjugated in appropriate amounts. One initial criterion is the stability of the core SPION in long term and throughout the conjugation steps which would require dilution and different pH conditions. For this purpose alkoxy silane based coatings including aminopropyltrimethoxysilane (APTMS) and carboxyethylsilanetriol (CES), alternatively meso-2,3-dimercaptosuccinic acid and poly(acrylic acid) (PAA) coated SPIONs were produced. Another key point is to obtain such particles in small size regime (less than 150 nm) so that the molecular targeting strategy can work. After initial assessment PAA coated SPION was found as the most successful, so this chapter focuses on the PAA-SPION based studies. Obtained results demonstrate that miR376b loaded SPIONs effectively target SKBR3 and MDA-MB-435 cell lines in vitro and in vivo, by showing effective miR376b transfection at miRNA and mRNA levels in vitro, demonstrating the first natural protein coated SPION miRNA delivery system in the literature.

3.2. Experimental

3.2.1. Materials

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were purchased from Merck (U.S.A.). Ammonium hydroxide (26%), poly(acrylic acid sodium salt), Bradford reagent, bovine serum albumin (BSA) standard and Potassium Phosphate dibasic anhydrous were purchased from Aldrich (U.S.A.). Phosphate buffer saline (PBS) and 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate buffers were purchased from Biomatik (Canada). Ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased from Multicell (U.S.A.). Potassium Phosphate monobasic was purchased from Riedel-de Haën (U.S.A.). Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo NHS), DyLight 650 NHS ester, Ellman's Reagent, Traut's Reagent and Slide-A-Lyzer 10k, 20k dialysis cassettes were purchased from Thermo Scientific (U.S.A.). antiHer2 was purchased from eBioscience (U.S.A.). Human AGO2 (His tag) (EIF2C2) recombinant protein was purchased from Sino Biological Inc. (China). antiHer2 (BMS 120) was purchased from Bio-Rad Antibodies. Maleimide and amine heterobifunctionalized polyethylene glycol (Mal-PEG- NH_2) (2k) was purchased from Nanocs (U.S.A.). Vivaspin 20 MWCO 30,000 polyethylenesulfonate (PES) filters were purchased from Sartorius (Germany). Float-A-Lyzer MWCO 300,000 was purchased from Spectrumlabs (U.S.A.). N-N dimethylformamide (DMF) was purchased from Merck-Milipore (U.S.A.).

3.2.2. Synthesis of polyacrylic acid coated SPIONs (SPION/PAA)

SPION/PAA was synthesized in water with the precursors given molar ratio: $[\text{PAA}]$: $[\text{Fe}^{2+}]$: $[\text{Fe}^{3+}]$: $[\text{OH}^-]$, respectively 3:2:1:6 under argon atmosphere and at 85°C for 1 h. After the reaction, mixture was placed on 0,3 Tesla magnetic field for overnight, then the precipitates were removed, and colloidal suspension was purified with ultracentrifugation using 10 kDa MWCO PES filter. Some amount of product was lyophilized for solid analysis.

3.2.3. Synthesis of AGO2 conjugated SPIONs (SPION/AGO2)

50 mg of SPION/PAA was activated by mixing with 14 mg of EDC and 14.4 mg of sulfo-NHS in MES buffer at pH 6.0 at room temperature for 15 min. Then, NHS active SPIONs were purified and exchanged to PBS, pH 7.4 using ultracentrifugation with 10kDa MWCO Amicon filter at room temperature for 30 min. After buffer exchange, 150 µg of AGO2 protein was added to SPIONs and mixed 48h at +4°C. Reaction was quenched with excess hydroxylamine and resulting mixture was purified with dialysis using 150k dialysis device (Float-A-Lyzer) for 12h at +4°C with 4 times buffer refreshment in MES buffer pH 6.0.

3.2.4. Activation of SPION/AGO2 with maleimide groups (SPION/AGO/MI)

SPION/AGO2 was activated and purified with EDC/sulfo NHS using the method given for SPION/PAA/AGO2 synthesis, then 3 mg of NH₂-PEG-Mal (2000 da) was added and mixed for 48h at +4°C and quenched with excess hydroxylamine. Product was purified using ultracentrifugation with 10kDa MWCO amicon filter at +4°C for 30 min.

3.2.5. Fluorescent tagging of antiHer2 (antiHer2-dye)

Commercially available NHS active dye (Dylight 650[®]) was dissolved in DMF (10mg/mL). antiHer2 antibody was dissolved in sodium borate buffer at pH: 8.55. Antibody and dye solutions were mixed in the molar ratio of [antiHer2] : [Dye] = 1:10 at room temperature for 1h. Overall product was purified with dialysis using 20 kDa MWCO dialysis cassette (Slide-A-Lyzer) against phosphate/EDTA buffer (pH:8.5) at +4°C during 12 h with 4 times buffer refreshment. Protein concentration and molar ratio of protein and dye was calculated the formulas in the following.

$$\text{antiHer2 Concentration (M)} = \frac{[A_{280} - (A_{655} \times 0.037)] \times \text{dilution parameter}}{210,000 \times 0.1 \text{ cm}}$$

$$\text{Dylight 655 per mol antiHer2 (mol)} = \frac{A_{655} \times \text{dilution parameter}}{73,000 \times \text{protein concentration} \times 0.1 \text{ cm}}$$

3.2.6. Thiolation of antiHer2 (antiHer2-Dye-SH)

antiHer2-Dye was mixed with Traut's Reagent at pH 8 with the molar ratio of [1:100] at room temperature during 2h, then resulting product was purified with dialysis from 10k MWCO dialysis cassette at +4°C for 4h with 6 times buffer refreshment in PBS/EDTA.

Sulfhydryl groups on antiHer2 were quantified using Ellman's reagent. 25 µL Ellman's reagent (1mg/mL) was mixed with 25 µL of antiHer2-dye-SH at room temperature for 15 min, then absorbance value at 412 nm was measured to obtain concentration of thiol moieties with Beer Lambert equation given in the following :

$$A = \epsilon \times b \times c$$

$$0.003 = 14,150 \text{ M}^{-1}\text{cm}^{-1} \times 0.1 \text{ cm} \times c$$

3.2.7. Synthesis of antiHer2-Dye conjugated SPIONs (SPION/AGO2/antiHer2-dye)

Freshly prepared antiHer2-dye-SH construct was mixed with maleimide functionalized SPION/AGO2 in PBS/EDTA at pH 7.2 for 1h at room temperature and overnight at +4°C then overall product was purified with the dialysis from 300 kDa MWCO dialysis device membrane at +4°C with 4 times buffer refreshment in PBS.

3.2.8. Determination of AGO2 concentration on SPIONs by Bradford Assay

Bovine serum albumin protein at 0, 1, 2, 4, 6, 8, 10, µg/mL in PBS was prepared to measure as reference standard (Adj. $R^2 = 0.9950$). Dialysis product (dialysate) of SPION/AGO2 was concentrated and measured as analyte. 1 ml of each reference and analyte were mixed with 1 mL of Bradford Reagent for 10 min at room temperature then absorbance at 595 nm was recorded using UV-Vis spectroscopy.

3.2.9. Determination of antiHer2 concentration on SPIONs by photoluminescence

Mole number of DyLight 650[®] on SPIONs was calculated to using calibration curve of PL vs concentration of DyLight 650[®]. Certain concentrations of dye (2×10^{-2} , 1×10^{-2} , 4×10^{-3} , 2×10^{-3} , 1×10^{-3} , 5×10^{-4} μM) were dissolved in PBS then PL intensities at 672 nm was recorded. Calibration curve of PL intensity vs concentration of dye was fitted to linear to obtain PL intensity vs concentration equation. (PL intensity = 3.01×10^8 DyLight 650[®] conc. + 1.2×10^5 , $R^2 = 0.998$.) Fluorescence intensity of SPION/AGO2/antiHer2-Dye was measured at 672 nm (1.8 M (a.u.), dilution factor : 20) and placed into the equation to find concentration of dye on SPIONs (0.114 nmol). To calculate concentration of antiHer2 on SPIONs, the result from mole of dye per mole of antiHer2 (given in the “Fluorescent tagging antiHer2” section) was used (antiHer2 : Dye = 1 : 0.8) and mole number of antiHer2 was found as 0.142 nmol. Using the molecular weight of antiHer2 (148,000 g/mol) concentration of antiHer2 on SPION dispersion was calculated as 19 $\mu\text{g/mL}$.

3.2.10. Quantification of number of AGO2 and antiHer2 per nanoparticle

Number of proteins per nanoparticle was calculated using the following equation

$$n = a \times N \times \frac{V}{\frac{4}{3} \pi r^3}$$

Where, a is the mole number of protein in 1 cm^3 , N is Avogadro's number, V is the volume of nanoparticles in 1 cm^3 and r is the mean radius of nanoparticles (z- average hydrodynamic size). V is calculated by subtracting volume of water in 1 cm^3 dispersion from the 1 cm^3 . Volume of water in 1 cm^3 dispersion was also found over mass of water in 1 cm^3 dispersion.

3.2.11. Cell culture

This experiment was conducted by Muhammed Koçak at the Molecular Biology

Department in Sabancı University. HEK293T human embryonic kidney cells were cultured

in Dulbecco's modified Eagle's medium (DMEM, Biological Industries, #BI01-050-1A) supplemented with 10% (v/v) fetal bovine serum (FBS, PAN, #P30-3302), antibiotics (Penicillin/streptomycin, Biological Industries, #BI03-031-1B) and L-Glutamine (Biological Industries, #BI03-020-1B) in a %5 CO₂ humidified incubator at 37°C. The breast cancer cell lines MCF-7 and MDA-MB-453 were cultured in DMEM medium supplemented with FBS, penicillin/streptomycin, L-Glutamine and SKBR3 cells were cultured in McCoy's 5A supplemented with FBS, penicillin/streptomycin, L-Glutamine. HEK293T was transiently transfected with calcium phosphate transfection method according to standard protocols.

3.2.12. Cytotoxicity assay

This experiment was conducted by Muhammed Koçak at the Molecular Biology Department in Sabancı University. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was applied to evaluate the toxicity of the nanoparticles complexes on the selected cells. MDA-MB-453 and SKBR3 cells were seeded onto 96-well plates and cultured with the same conditions as follows : cells were treated nanoparticles for 24 and 48 hours after 24 h cultured time. Then, 10µl of 5mg/ml MTT reagent was added into 100µl of cell medium and incubated for 4 hours in a %5 CO₂ humidified incubator at 37°C. At the end of 4 hours of incubation, the medium was removed and formazan crystals were solubilized with dimethyl sulfoxide (DMSO) for 10 minutes at room temperature. The absorbance at 570 nm was determined with a reference filter at 650 nm for each well using a Microplate absorbance reader (Biorad, iMark). Cell viability of each group was expressed as a percentage of the viability of untreated control cells.

3.2.13. Fluorescence microscopy imaging

This experiment was conducted by Muhammed Koçak at the Molecular Biology Department in Sabancı University. Cells were cultured on coverslips and treated with 150µg

of nanoparticles for indicated time periods, then fixed in ice-cold 4% paraformaldehyde/PBS. For colocalization experiments, cells were co-transfected with GFP-Rab9 and pmTurquoise2-Mito plasmids. 48 hours later, cells were fixed in ice-cold 4% paraformaldehyde/PBS. Following fixation, nuclei were stained using Hoechst (Invitrogen, 31716W) in PBS. Coverslips were mounted onto glass slides, and samples were analyzed using a Carl Zeiss LSM 710 confocal microscope (Zeiss, Germany).

3.2.14. *In vivo* experiments

These experiments were conducted by Muhammed Koçak and Yunus Akkoç at the Molecular Biology Department in Sabancı University. Nude mice were intravenously injected with (10 mg/kg Fe^{+2}) nanoparticles were intravenously injected and inoculated for indicated time periods (8 hours, 10 days and 40 days). At the end of inoculation, blood samples were collected by intracardiac puncture under anesthesia and serum biochemical parameters (ALB, ALKP, ALT, AMYL, Ca, GLU, LIPA, TBIL, TP, BUN and GLOB) were analyzed by using diagnostic health profile kit (IDEXX VetTestTM, #6802590) on an Olympus AU400 according to manufacturer's instructions.

A xenograft mouse model was generated by subcutaneous injection of SKBR3 and MDA-MB-453 cells (1×10^7 cells per mouse embedded in Matrigel (BD Biosciences)) in the right front flank of female nude mice (6 - 8 weeks, 20 g). When the tumors reached $\sim 50\text{-}100 \text{ mm}^3$, approximately 8-10 weeks after inoculation, the tumor bearing mice were injected via tail vein a single dose of nanoparticles (10 mg/kg Fe^{+2}). After 24 hours of injection, *in vivo* imaging of the nanoparticles was conducted under isoflurane inhalation anesthesia by using IVIS Spectrum imaging system (Caliper Life Sciences) with the Living Image software package. Anesthetized mice were euthanized through cervical dislocation and dissected to

remove tumors, livers, brain, intestine, stomach, uterus, kidneys, spleens, hearts, and lungs which were placed into 10% Formalin. Fluorescence from each organ was imaged.

3.2.15. Loading of therapeutic miRNAs into the SPION-protein bio-conjugates

This experiment was conducted by Muhammed Koçak at the Molecular Biology Department in Sabancı University. Nanoparticles were incubated in RNA binding buffer (100mM KCl, 2nM MgCl₂, 10mM Tris-HCl in DEPC water) together with 20nM miR376b mimics at 4°C for 2 hours on rotator. 10µg of protein extract was added in RBB buffer before incubation miRNA mimics to use the ability of endogenous RNA-induced silencing complex (RISC) elements to bind miRNA to AGO2 protein on nanoparticles.

3.2.16. RNA isolation and RT-PCR analyses

This experiment was conducted by Muhammed Koçak at the Molecular Biology Department in Sabancı University. Total RNA was extracted using TRIzol reagent (Sigma-Aldrich, #T9424) according to the manufacturer's instructions. cDNA was reverse transcribed from DNase-treated total RNA using M-MuLV reverse transcriptase (Fermentas, #EP0351), random hexamers (Invitrogen, #48190-011) and miR376b stem-loop primer.

For single step qRT-PCR reaction, SYBR Green Quantitative RT-PCR kit (Roche, #04-913-914-001) and an LightCycler 480 (Roche) were used. To activate the SYBR green, an initial cycle of 95°C, 10 min was performed followed by PCR reactions: 40 cycles of 95°C for 15 sec. and 60°C for 1 min. Then a thermal denaturation protocol was used to generate the dissociation curves for the verification of amplification specificity (a single cycle of 95°C for 60 sec., 55°C for 60 sec. and 80 cycles of 55°C for 10 sec). Changes in mRNA and miRNA levels were quantified using the $2^{-\Delta\Delta CT}$ method using GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA and U6 small nuclear 1 (RNU6-1) mRNA as control. Primers used during the study were: BECN1 primers 5'-AGGTTGAGAAAGGCGAGACA-3'; 5'-

GCTTTTGTCCACTGCTCCTC-3'; ATG4C primers 5'-GCATAAAGGATTTCCCTCTTGA-3'; 5'-GCTGGGATCCATTTTTCG-3', and GAPDH primers 5'-AGCCACATCGCTCAGACAC-3'; 5'-GCCCAATACGACCAAATCC-3'.

TaqMan qRT-PCR reactions were performed using FastStart Universal Probe Master kit (ROCHE, #04913957001) and LightCycler 480 (Roche) according to the protocols described previously. Primers and the probe used during the study were: Stem-loop primer, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACATGG-3'; Forward primer, 5'-GTTAATCATAGAGGAAAAT-3'; Reverse primer, 5'-GTGCAGGGTCCGAGGT-3'; TaqMan Probe, 5'(6-FAM)-GCA GGG GCC ATG CTA ATC TTC TCT GTA TCG -(TAMRA-sp)³'; U6 forward primer, 5'-CTCGCTTCGGCAGCACA-3'; U6 reverse primer, 5'-RAACGCTTCACGAATTTGCGT-3'; U6 TaqMan Probe 5'(6-FAM)-GCA GGG GCC ATG CTA ATC TTC TCT GTA TCG-(TAMRA-Sp)³'.

3.2.17. Statistical analyses

This experiment was conducted by Muhammed Koçak at the Molecular Biology Department in Sabancı University. Statistical analyses were performed using Student's two-tailed t-test. Data were represented as means of $\pm$ SD of n independent experiments (biological replicates). Values of $p < 0.05$ were considered as being significant. Reactions were performed in duplicates and number of independent experiments (n) was marked.

3.3. Characterization Methods

Photoluminescence spectroscopy (PL) measurements were performed with Horiba Jobin Yvon FluoroMax-3 at room temperature. Absorbance data were collected with UV-3600 Shimadzu UV-VIS-NIR spectrophotometer at room temperature. Dynamic light scattering (DLS) experiments were conducted with Zetasizer Nano Series ZS at room temperature with 173° backscattered angle. Functional groups were investigated

using the dried samples and Thermo Scientific Nicolet iS10 ATR-IR. Transmission electron microscopy (TEM) images were taken by Techani G2 F30 at brightfield high resolution (HR) TEM (acceleration voltage 200 kV) using samples deposited on carbon coated Cu-grid from dilute solutions. Vibrating Sample Magnetometry (VSM) measurements were conducted with Cryogenic limited PPMS at ambient conditions. Thermogravimetric analysis (TGA) was made by using TA Q500 under nitrogen atmosphere. X-ray diffraction (XRD) patterns were recorded with Bruker D8 Advance X-ray Diffractometer using Cu-K α radiation.

3.4. Results and Discussions

In this study we aimed to generate versatile and highly biocompatible magnetic nanoparticles by introducing functional proteins antiHer2, AGO2 and a fluorescent dye in order to target breast cancer, deliver therapeutic miRNAs, and follow particles based on fluorescence imaging *in vitro* and *in vivo*. During the synthesis it was critical to carefully conjugate each component to SPION surface (PAA) in appropriate order and in sufficient amounts in order not to hinder or prevent the conjugation of others and to provide sufficient amounts of both AGO2 and antiHer2. Besides, maintenance of colloidal stability of particles during the conjugation chemistries in a wide pH range is an important limitation yet must be achieved. With these considerations, several SPIONs with different surface coatings were prepared and tested for AGO2 and antiHer2 conjugation in separate reactions to choose the most appropriate SPION core and to optimize the chemical methods for the generation of ultimate multifunctional nanoparticle composition. As explained in the introduction of this chapter, the following sections will summarize the preparation of the target nanoparticle using PAA coated SPION which provided the most successful synthetic methodology.

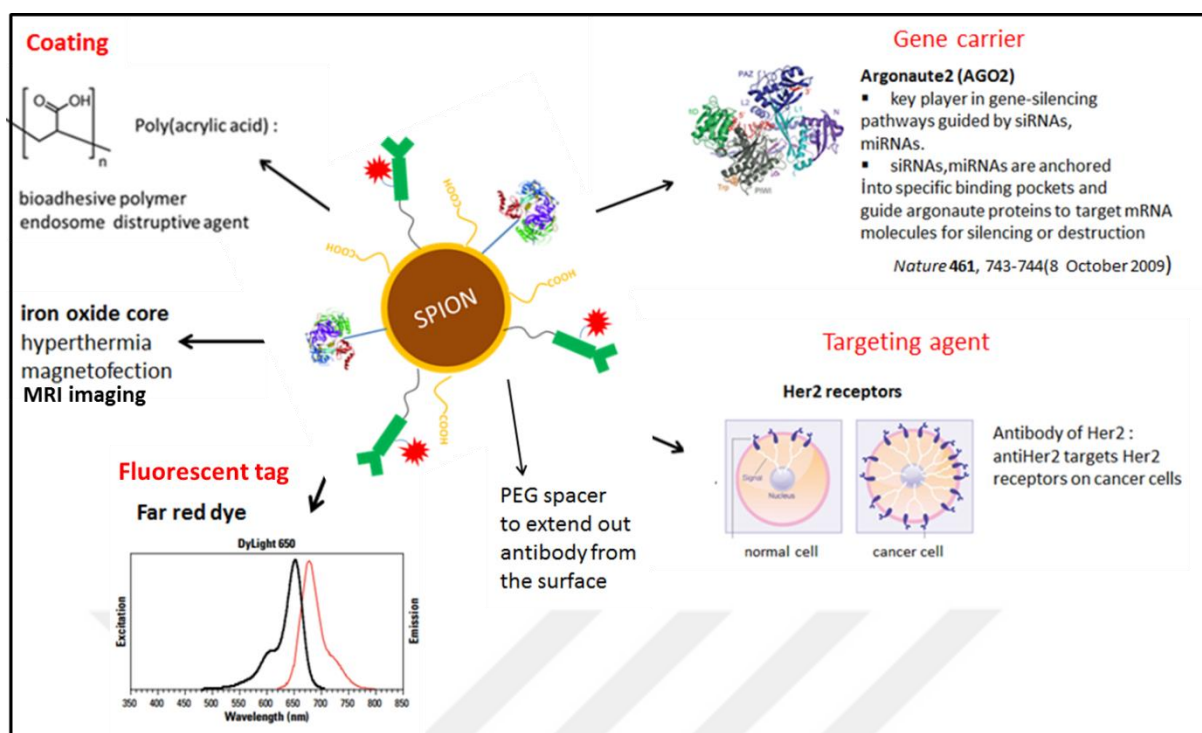
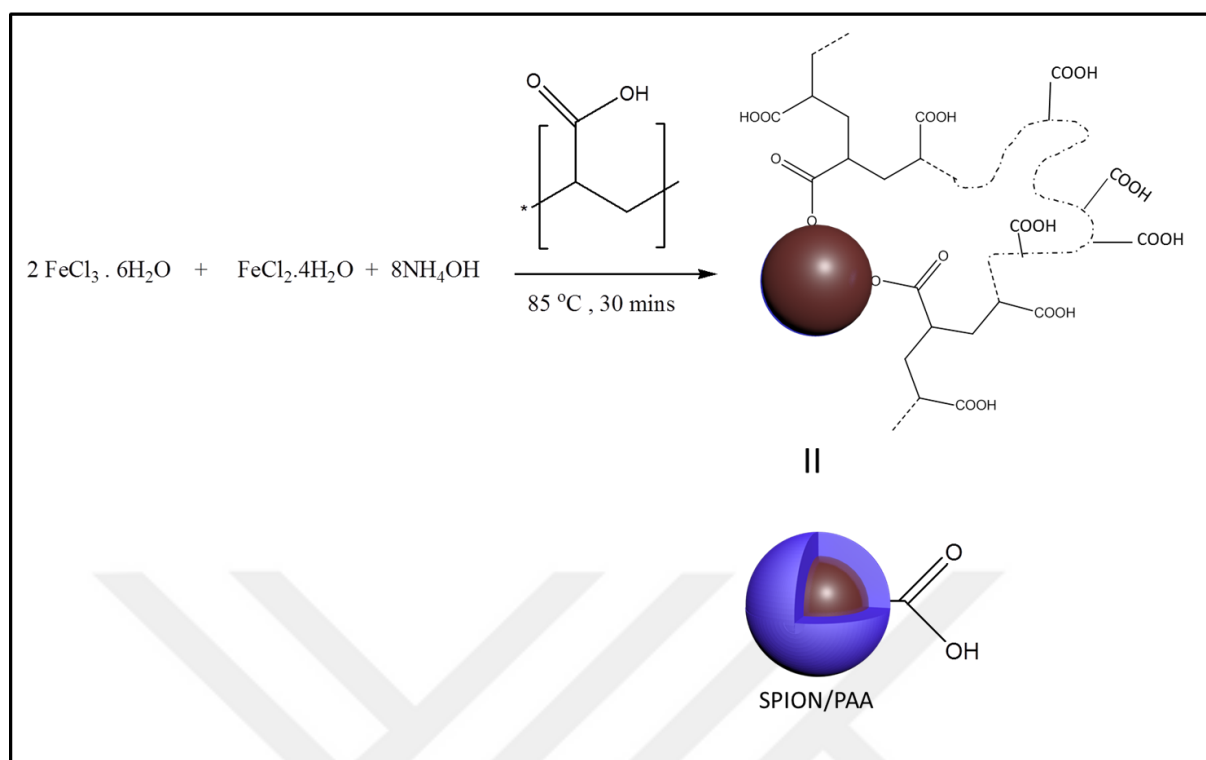


Figure 3. 1 Representative design of the protein coated multifunctional SPIONs

3.4.1. Synthesis and Characterizations of Functional SPIONs

In order to obtain water soluble and biocompatible SPIONs for miRNA delivery applications, SPION/PAA were synthesized directly using aqueous co-precipitation method from the ferric and ferrous salts under alkaline conditions (Figure 3.2). TEM micrograph shows the crystal size of these particles as about 4 nm without a noticeable aggregation (Figure 3.2a). Colloidally stable particles have about 17 nm hydrodynamic size which falls into the ultra-small size regime and have -19 meV zeta potential as expected from a carboxylic acid functional surface (Figure 3.2 b-c).



Scheme 3. 1 Aqueous synthesis of SPION/PAA.

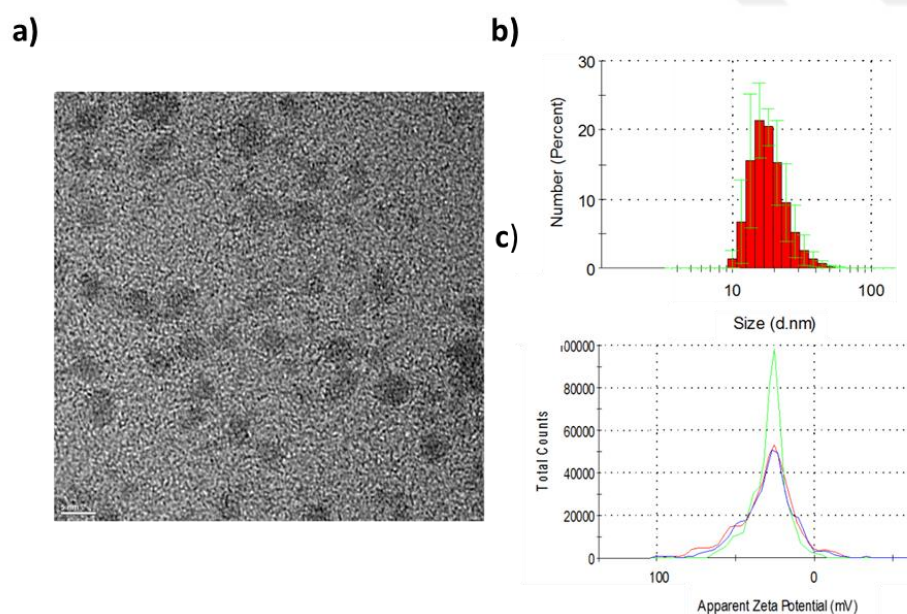


Figure 3. 2 (a) TEM micrograph of SPION/PAA (Scale bar : 10 nm) (b) Number average hydrodynamic size distribution and (c) zeta potential distribution measured by DLS (in water)

XRD diffraction patterns of SPION/PAA nanoparticles indicate a spinel structure of magnetite (Fe_3O_4) (Figure 3.3 a). Broad peaks in the XRD is attributed to the small size of the crystals. Magnetization of SPION/PAA in applied magnetic field indicates superparamagnetic nature with zero hysteresis and high magnetization value (Figure 3.3b). PAA layer on SPION was verified through comparative FTIR analysis of PAA, SPION/PAA and bare SPION (Figure 3.3 c). When compared SPION/PAA with bare SPIONs, effective coating of PAA on nanoparticles was observed.

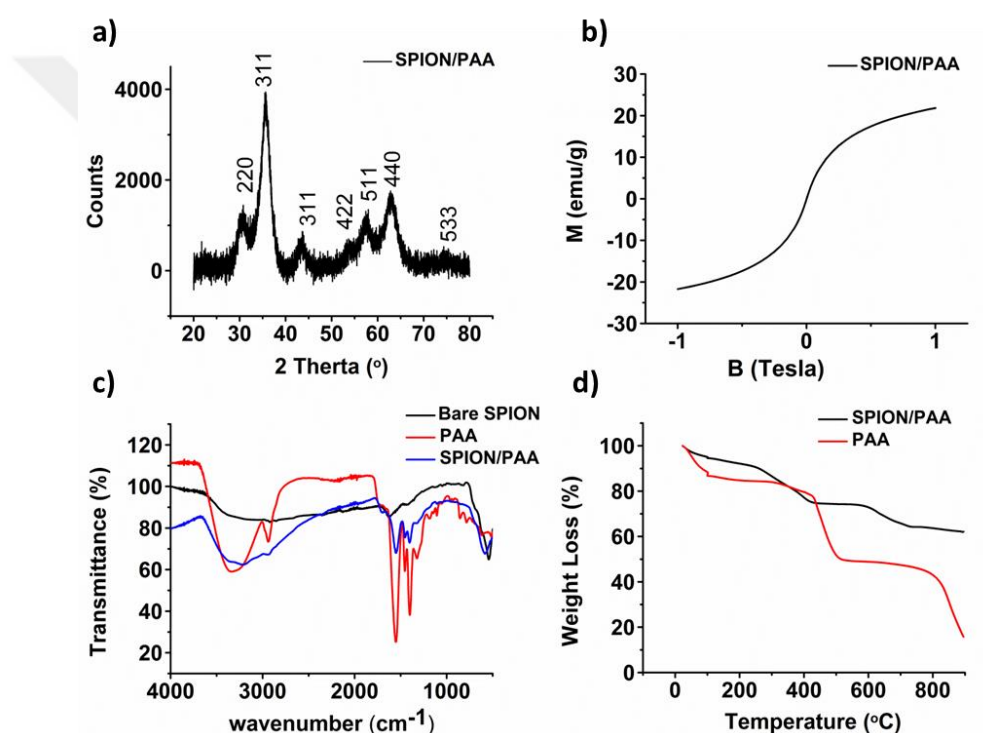


Figure 3. 3 a) Powder XRD pattern of SPION/PAA (indexed as cubic magnetite) (B) Magnetization (M) as a function of applied magnetic field at 300 K. c) FTIR spectra of bare SPION, PAA and SPION/PAA d) Thermogravimetric analysis of SPION/PAA and PAA.

Strong symmetric stretching of carbonyl group (1570 cm^{-1}) and broad band of hydroxyl groups ($2800 - 3700\text{ cm}^{-1}$) on the spectrum of SPION/PAA confirms the presence of carboxylic acid functional groups. Band at 584 cm^{-1} is the symmetric stretching of Fe-O

interacting with PAA. Thermogravimetric analysis indicates a two stage degradation and about 36% organic content for SPION/PAA. (Figure 3.3 d)

3.4.2. General Considerations for the Design of SPION-Protein Bio-conjugates

Bio-conjugation is an emerging approach to create new and smart bio-active constructs. Bio-conjugate chemistry includes various strategies to attach biomolecules onto the chemically active surfaces. [268] Consideration of the ultimate application of the bio-conjugate is the most significant aspect in the bio-conjugation. In order to obtain a proper design, there should be enough knowledge about the structural and chemical properties of each building block. Hence, how these modifications can potentially affect the activity of selected biomolecules has to be taken into account during the bio-conjugation strategies.

In this project, functional proteins, antiHer2 and AGO2, have to be conjugated onto the surface of SPION. This means they will be conjugated to the carboxylic acid of the PAA coating of the SPION. The three dimensional structure, functionality, active site and stability of these proteins under the synthetic conditions and after conjugation were taken into consideration to prevent loss of their desired function and if possible obtain maximum performance from each component in the final composition. For this purpose, one of the critical factors is thought as the distance of these proteins from the core of the nanoparticle. Since antiHer2 is used to interact with Her2 receptors on breast cancer cells, this protein has to be far enough from the SPION surface and from the steric crowding of the AGO2 (which is also conjugated to the surface) in order to achieve a favorable receptor-antibody interaction. Therefore, antiHer2 was attached to SPIONs with a relatively longer spacer (39.7 Å) compared to the size of AGO2. Since AGO2 is used to carry the therapeutic miRNAs in its binding pocket and protect it from the enzymatic degradation during the circulation, AGO2 was kept closer to the SPION surface and was conjugated via direct amide linkage.

Three dimensional structures and functionalities of antiHer2 and AGO2 were given in the Figure 3.4. antiHer2 is a standard, Y-shaped human immunoglobulin (IgG) containing Fab and Fc fragments.[269] Antigen binding sites of an IgG is usually found in the top of the Fab fragments. These sides have to be preserved during the bio-conjugation steps. The most common side of antibodies for bio-conjugation is their primary amine units, because they are abundant on the surface, especially on the lysine residues, and they have high reactivity. Unfortunately, lysine residues are located predominantly on the antigen binding site and this would create a hindrance for an effective receptor targeting. In order to overcome this limitation, density of attachment per antibody should be keep at the minimum level, yet, should be high enough to provide receptor-mediated endocytosis of nanoparticles, which is a second critical design strategy.

AGO2 is a globular protein consisting of four distinct units : the N terminal, PAZ, Mid, PIWI. AGO2 has a significant role in mRNA degradation; it was reported that PIWI domain is enzymatically active while PAZ and MID domains do interact with small interfere RNAs.[242] The role of AGO2 in this work is to carry therapeutic miRNA into the tumor cells or tissue. However, there is not any example in the literature for the conjugation of AGO2 and it is not known which reactive units on the protein responsible for the interaction with miRNAs. Solely, it is shown that AGO2 complexed with miRNAs through its binding pockets, therefore we assumed that occupation of some active amine units on the protein would not probably show a significant reduction in its functionality. Hence, after the development of SPION-AGO2 bio-conjugates, we also examined this assumption by *in vitro* and *in vivo* tests. In this thesis work, AGO2 protein was attached via its amine units to the carboxylic acid of the SPION/PAA in a simple one step reaction utilizing the activated ester approach (Scheme 3.2).

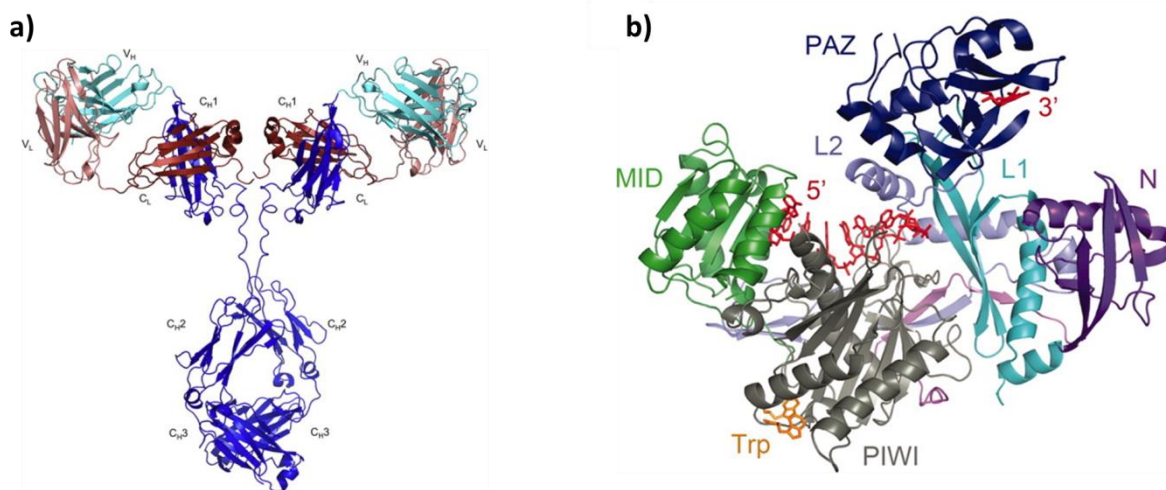


Figure 3. 4 a) Structure of a human IgG [269] and b) human AGO2 [242]

Since proteins are sensitive to temperature and pH, gentle synthesis and purification protocols were applied for their conjugation to SPIONs. antiHer2 and AGO2 conjugation reactions were carried out in the range of physiological pH and at +4 °C in overnight or longer reaction times.

The typical approach to create bio-conjugates is to use reactive crosslinking agents. The key point to create a successful and active bio-conjugate is to select an appropriate crosslinking agent which can react with the active groups of both molecules that has to be linked together and preserve the colloidal stability.[268] This is called as a crosslinking in the field of bio-conjugation and mean linking to building blocks via a linker not crosslinking a matrix as known in polymer chemistry. In the selection of these crosslinking agents, one important consideration was to keep particles always suspended in the buffer solutions in which conjugation reactions are performed, hence hydrophilic linkers are preferred. Such linkers may have homofunctional or heterofunctional end groups specific for conjugation with typical functionalities such as carboxylic acid, amine, thiol and an aldehyde groups. Mostly used crosslinking agents for the protein conjugation are given in the Table 3.1.

Table 3. 1 Mostly used reactive groups for protein conjugation.[268]

Reactive Group	Chemical Group
Carboxyl to amine reactive groups	Carbodiimide (EDC)
Amine reactive groups	NHS ester, imidoester
Sulfhydryl reactive groups	Maleimide, haloacetyl, Pyrldyl disulfide, thiosulfonate
Aldehyde reactive groups	Hydrazide, alkoxyamine
Photoreactive groups	Diazirine, aryl azide
Hydroxyl reactive groups	Isocyanite

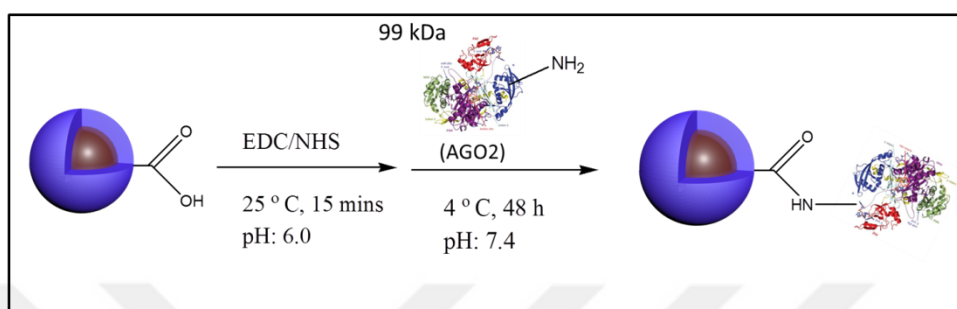
With all these synthesis condition considerations mentioned above, and desire to not to expose AGO2 to too many different solvents in a multi-step synthesis, AGO2 was conjugated directly from its surface amines to carboxylic acid of SPION/PAA via carbodiimide chemistry allowing a facile amid bond formation. For the conjugation of antiHer2, thiolation of antiHer2 and then its conjugation to PEG linker via thiol-maleimide chemistry was preferred. This conjugation is also widely used in protein chemistry since it is a highly specific and facile route for protein binding and gentle reaction conditions.

Multifunctional SPION-Protein bio-conjugates were carefully designed and synthesized under these given physical and chemical considerations. The details in the synthesis and characterization are summarized in the following sections.

3.4.3. Synthesis and Characterization of AGO2 conjugated SPIONs

In our design, AGO2 protein was covalently attached to SPIONs via amide bond using the carbodiimide (EDC/NHS) chemistry and activated ester approach to facilitate the reaction of carboxylic acids with the amine groups of AGO2 (Scheme 3.2). EDC

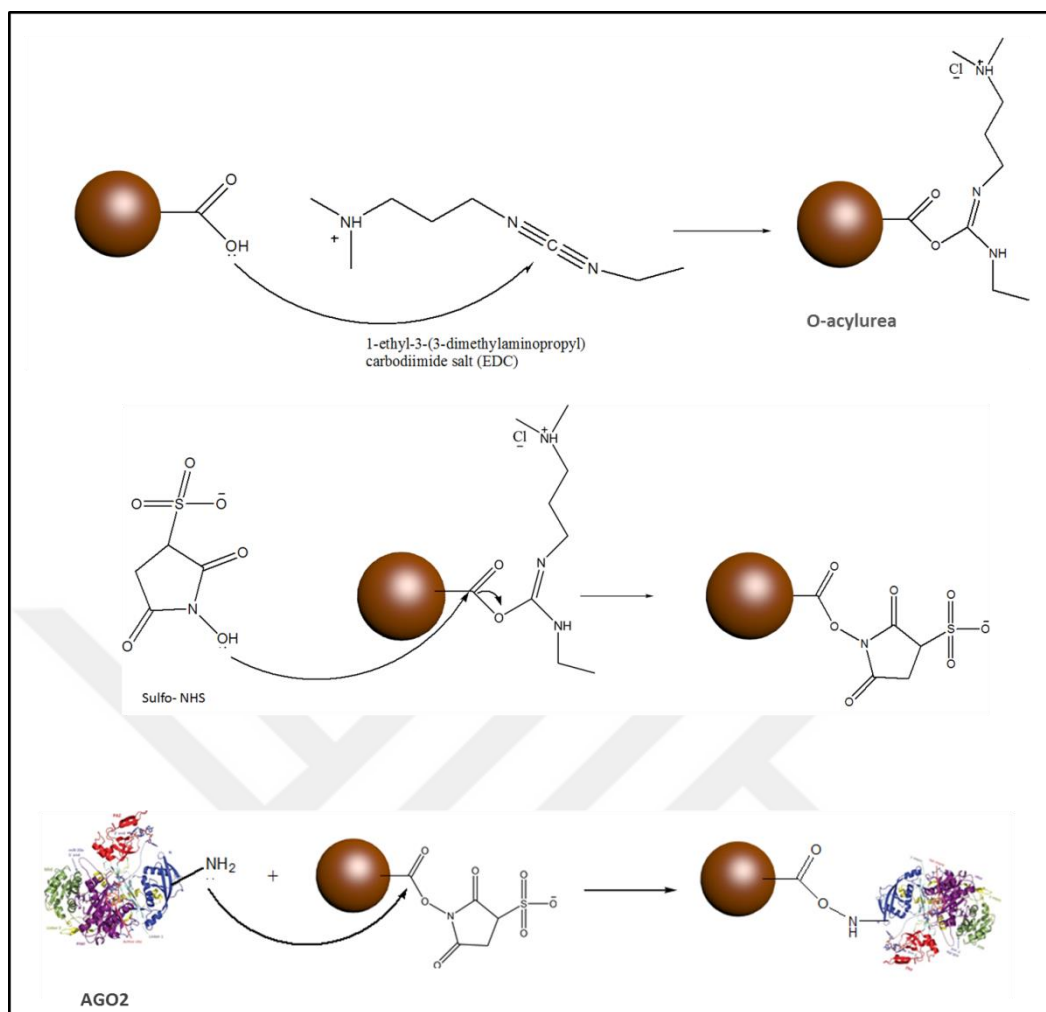
((ethyl(dimethylaminopropyl)carbodiimide) activates carboxylic acids by forming an o-acylurea which reacts fast with the NHS (N-hydroxysuccinimide) forming an activated ester with good leaving group and hence provides a facile reaction with the amine groups of the protein.



Scheme 3. 2 Synthesis of the AGO2 conjugated SPIONs (SPION/AGO2)

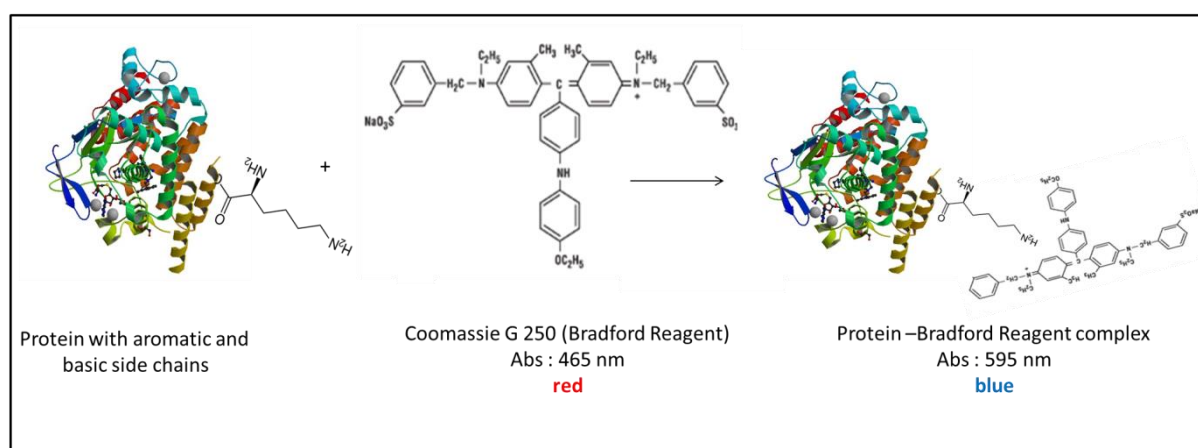
Since bulky proteins cannot be easily directed onto the o-acylurea, NHS ester is preferred before protein is introduced. The byproduct of this reaction, isourea, can be easily removed in aqueous washing steps via dialysis. Here, sulfonate group functionalized NHS was (sulfo-NHS) also used to prevent the aggregation of SPIONs when converted to NHS ester. pH is the most significant parameter for the efficiency of this reaction. Optimum pH for EDC conjugation is 3-4 in order to generate carboxylate from the carboxylic acid groups of PAA (pKa is around 4) of SPION/PAA. Since the activated particles were more stable in the pH range of 6-6.5, this reaction was conducted at pH 6 both to improve its conjugation and stability at the same time. Amidation reaction between the NHS ester of SPIONs and amine groups was carried out most efficiently at pH 7.2, because at this pH all primary amines of AGO2 deprotonated and contain reactive lone pair.

Typically, this reaction takes about 2 h at room temperature but in order to protect AGO2 from any kind of degradation, this reaction was conducted at +4°C for 48 hours. SPION/AGO2 was cleaned from all reagents by dialysis.



Scheme 3. 3 Synthesis mechanism of the SPION/AGO2

Bradford Assay was used in order to quantify the AGO2 content of the washed nanoparticles. Bradford assay is a protein detection method which uses the Coomassie Brilliant Blue G-250 dye as an indicator. This dye has red color in its cationic form ($A_{\text{max}} = 465 \text{ nm}$), but when it interacts with a protein it is converted into a non-protonated stable form which has blue color ($A_{\text{max}} = 595 \text{ nm}$). Protein amount can be calculated based on its absorbance at 595 nm. (Scheme 3.4)



Scheme 3. 4 Working mechanism of the Bradford Assay.

The absorbance values of the reference protein bovine serum albumin (BSA) are given in the Table 3.1. A calibration curve was plotted in Figure 3.4 based on these $A_{\max}$ values.

Table 3. 2 $A_{\max}$ values of BSA reference proteins at 595 nm

Concentration of BSA reference ($\mu\text{g/mL}$)	$A_{\max}$ (595 nm)
10	0.717
8	0.707
6	0.653
4	0.589
2	0.546
0	0.475

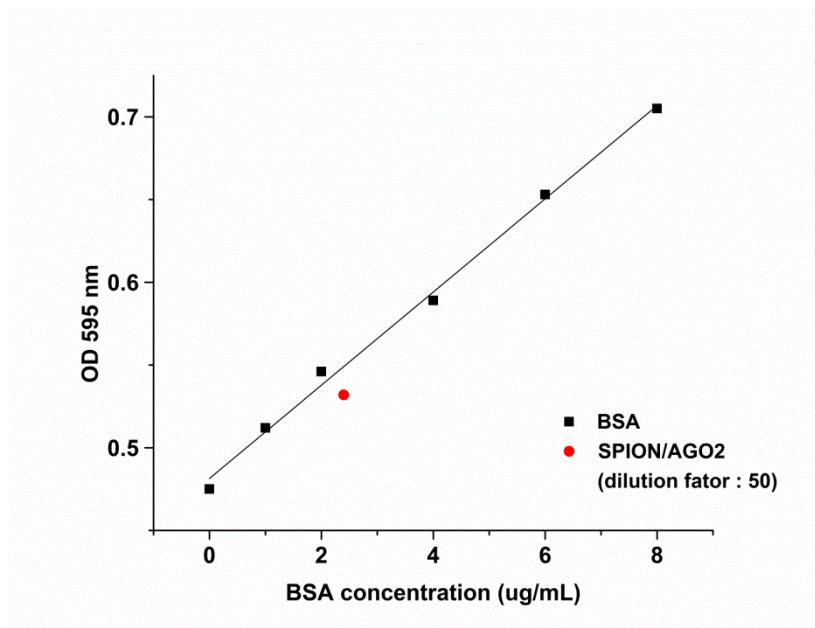


Figure 3. 5 Quantification of AGO2 by Bradford assay. Standard curve of BSA concentration ($\mu\text{g/mL}$) versus absorbance at 595 nm.

AGO2 concentration of the SPION/AGO2 solution was calculated as $66 \mu\text{g/mL}$ ($5.5 \mu\text{g}$ AGO2 per mg of nanoparticles) corresponding to nearly 124 AGO2 proteins per each nanoparticle (Table 3.2).

Table 3. 3 Amount of AGO2 on SPIONs quantified by Bradford Assay.

Sample	A_{max} at 595 nm	Dilution Factor	Amount of AGO2 per mg of NP (μg)	Number of AGO2 per NP
SPION/AGO2	0.532	50	5.5	124*

*Calculated by the formula given in the section 3.2.10

An important problem here is the absorbance of SPIONs themselves at 595 nm which would cause to the overestimation of the AGO2 concentration based on the absorbance of SPION/AGO2 at this wavelength. Therefore, unbound amount of AGO2 existing in the clear dialysate was also analyzed using Bradford Assay to calculate the amount bound in an indirect way. Bounded AGO2 was calculated as $118 \mu\text{g}$ via the Bradford assay while

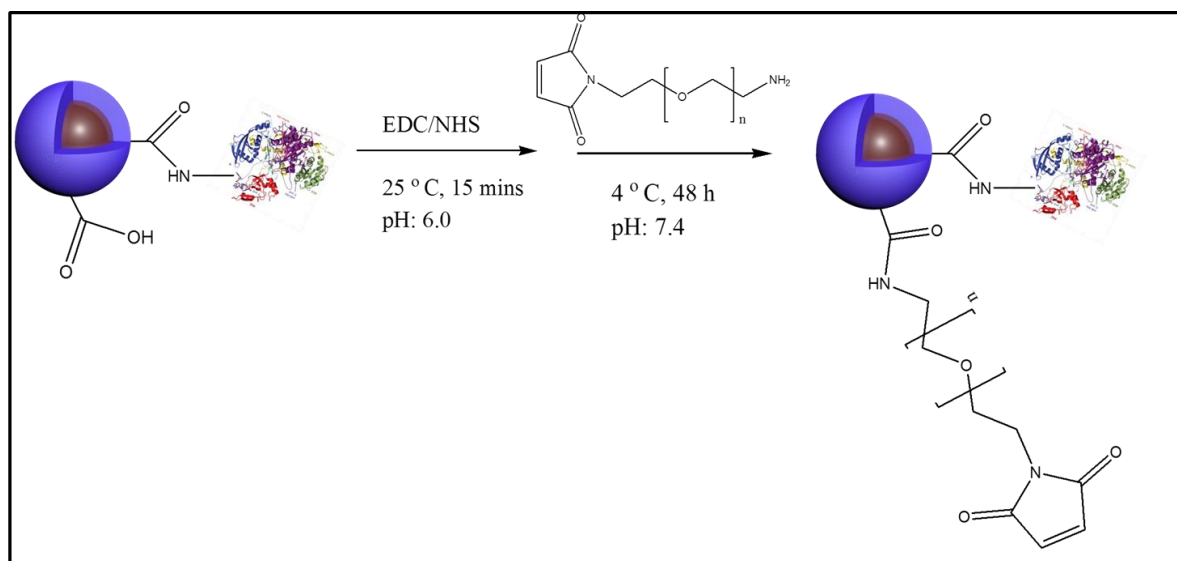
unbounded amount of AGO2 was calculated as 20 μg . Total amount of AGO2 added to the reaction mixture was 150 μg which shows that amounts of both the bounded and unbounded AGO2 calculations are in an agreement (Table 3.3).

Table 3. 4 Comparison of AGO2 amounts in the sample and dialysate by Bradford Assay.

Sample	A _{max} at 595 nm	Dilution Factor	Total Amount of AGO2 (μg)	Added Amount of AGO2 (μg)
SPION/AGO2	0.532	50	118	150
SPION/AGO2 dialysate	0.706	1	20	

3.4.4. Synthesis and Characterization of antiHER2 Conjugated SPIONs

The coupling reaction between antiHer2 and the nanoparticle surface was carried out with heterobifunctional amine and the maleimide active PEG bridge with 39.7 Å spacer length which has 8 repeat units which can also provide enough hydrophilicity and hence colloidal stability to particles in the intermediate steps. The use of hydrophilic and hydrophobic linkers can dramatically influence the stability and reactivity of the constructed bio-conjugate. [270] Besides, as explained in the introduction part, PEG moieties shield the nanoparticles from the RES system in the body. Therefore, using PEG spacers is a highly smart approach in terms of colloidal stability and pharmacokinetics. The amine groups of the PEG linker was attached to SPIONs again via EDC/NHS chemistry forming an amide bond Scheme 3.2. This provides maleimid (MI) functional nanoparticles (SPION/AGO2/MI) which can conjugate thioleated antiHeer2 to nanoparticles via thiol-ene chemistry (Scheme 3.5).



Scheme 3. 5 Synthesis of the MI functional SPIONs (SPION/AGO2/MI) with Amine-PEG-MI spacer

In order to track the nanoparticles *in vitro* and *in vivo*, nanoparticles were labelled with a fluorescent dye. However, the selection of the dye and the position that will be conjugated on the particle are critical issues. Since iron oxide nanocrystals have a strong absorbance between 300-650 nm (Figure 3.6 a), luminescence of a dye emitting in this range does quench significantly which would limit the detectability for the particles via optical means. Therefore, a far red dye (DyLight 650[®]) with emission maxima at 672 nm was used in this study (Figure 3.6 b). Besides, in order to track particles *in vivo*, dyes with emission in the far red or near infrared region are needed to suppress the tissue autofluorescence and have deeper penetration. In addition, in order to increase the distance between the SPION and the dye, conjugation of dye to the antiHer2 was planned. This strategy was followed to provide strong optical signal from dye-labelled nanoparticles with a relatively long shelf life.

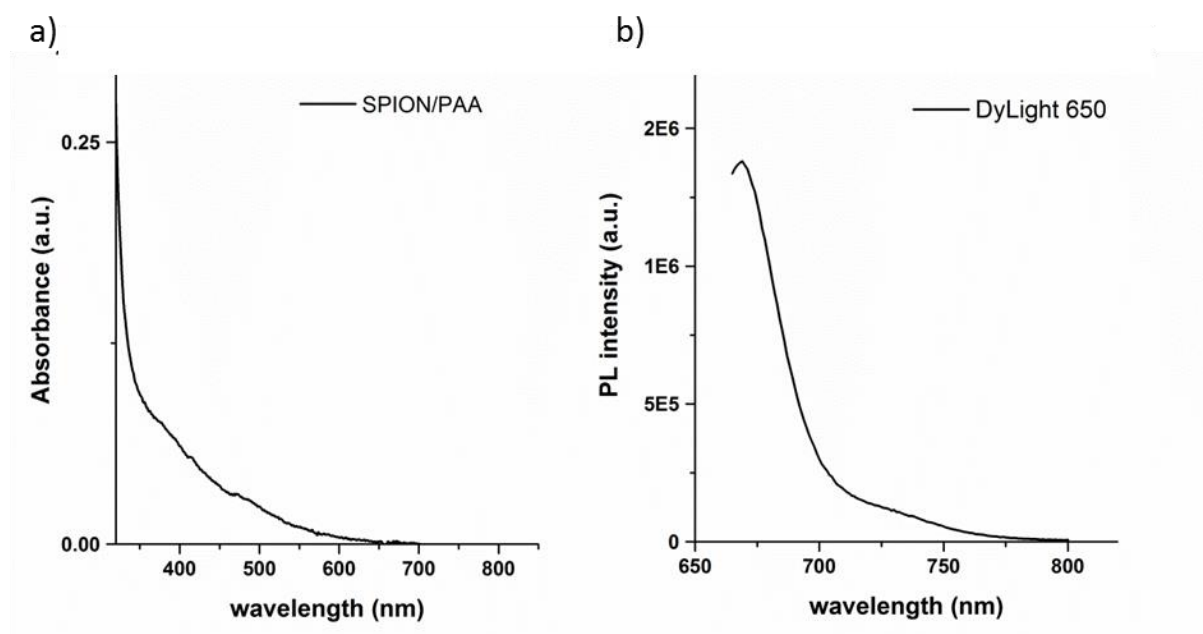
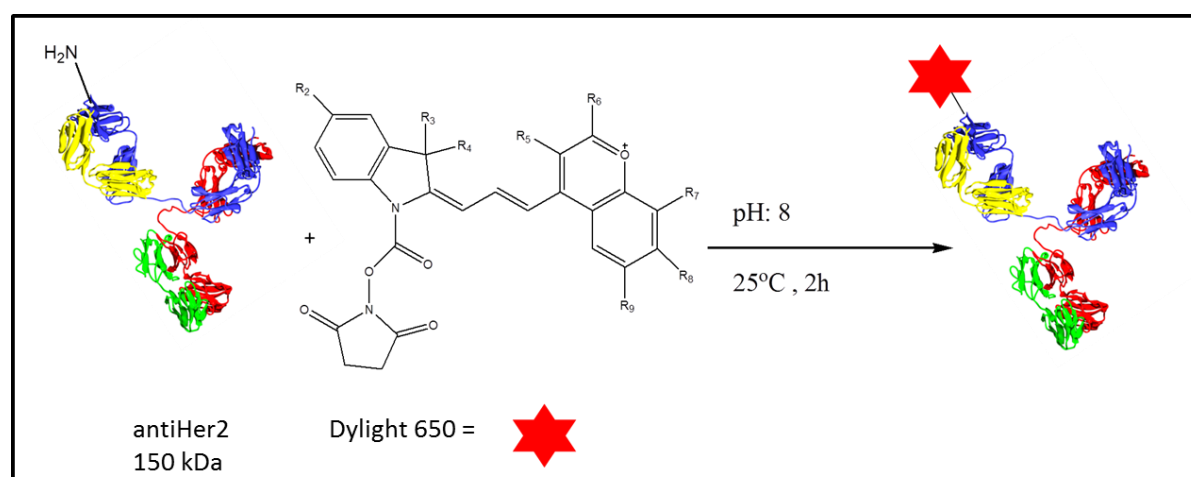


Figure 3. 6 a) Absorbance spectrum of SPION/PAA in PBS (0.08 mg/mL) b) PL spectrum of DyLight 650[®] in PBS (4 nM).

antiHer2 was fluorescently modified before conjugation onto the SPION/PAA. antiHer2 was labeled with NHS active DyLight 650[®] was used to conjugate it to antiHer2 from its amine groups via an amide bond (Scheme 3.6)



Scheme 3. 6 Synthesis of the DyLight 650[®] tagged antiHer2 (antiHer2-Dye).

In order to improve the yield of the reaction, dye was added as 10 fold molar excess of antiHer2. However, a crucial point at this step is not to attach to many dyes to antiHer2 which may reduce its affinity to the Her2 receptor. One mole of dye molecule per mole of antiHer2 is sufficient for imaging purposes. High concentration of dye on antiHer2 may also cause dye-dye interaction and reduce emission intensity. The amount of dye per mole of antiHer2 was calculated calorimetrically using the Beer Lambert equation (9) where A is the absorbance at the peak max, ε is the extinction coefficient, C is the concentration of the analyte and b is the pathlength.

$$A = \varepsilon \cdot b \cdot C \quad (9)$$

Absorbance at 280 nm is typically used for proteins so, absorbance at this wavelength was used to quantify the antiHer2 content of the particles using equation 10.

$$\text{Protein Concentration (M)} = \frac{A(280) - [A(655) \times 0.037]}{280,000 \text{ M}^{-1} \text{cm}^{-1} \times 0.1 \text{ cm}} \quad (10)$$

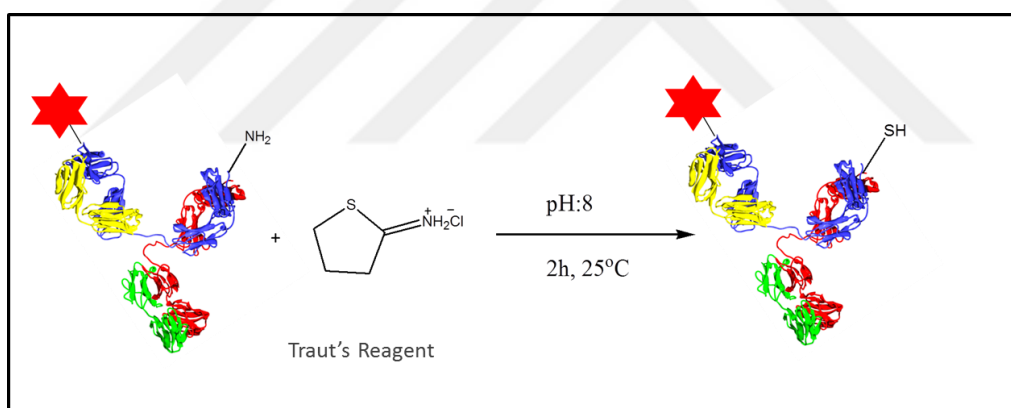
The molar ratio between Dylight 650[®] and antiHer2 was calculated with equation (11). Nearly 0.8 mole of dye molecule was attached per mole of antibody which was calculated using Beer Lambert equation (Table 3.5).

$$\frac{\text{Dylight 655 (mol)}}{\text{Protein (mol)}} = \frac{A(655) \times \text{dilution factor}}{73,000 \times \text{protein concentration}} \quad (11)$$

Table 3. 5 Mole of Dylight 650[®] per mole of antiHer2

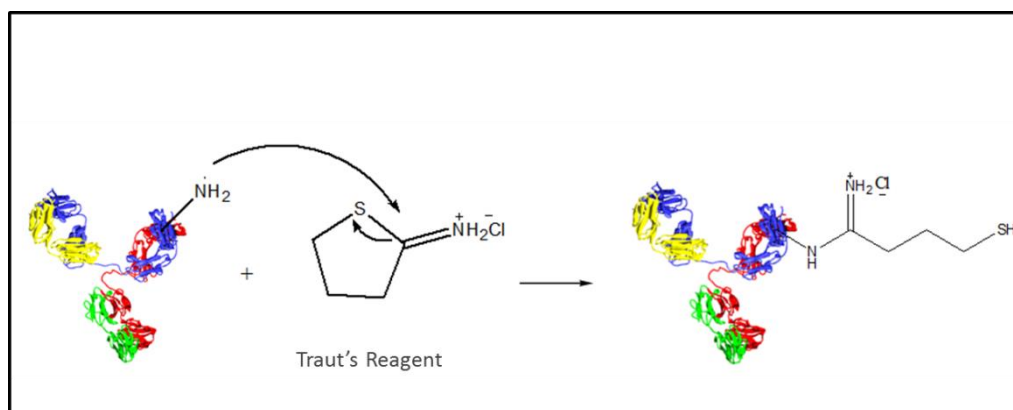
	A _{max} at 280 nm (antiHer2)	A _{max} at 655 nm (Dylight 650 [®])	Mol of dye per mol of protein
antiHer2-Dye	0.104	0.147	0.8

Before the thiol-ene conjugation of antiHer2-Dye with SPION/AGO2/MI, antiHer2 has to be thiolated. The most efficient way for thiolation of proteins is using the Traut's Reagent. Traut's Reagent is a cyclic thioamide compound (2-iminothiolane) which converts amines to sulfhydryl units (thiols) in a single step reaction.[271] Traut's Reagent reacts spontaneously with primary amines at pH 7-9. Amine containing buffers significantly decrease the stability of Traut's Reagent. For instance, half-life of Traut's Reagent in tertiary amine buffers is around 2 hours, while its half-life in primary amine containing buffer is nearly 5 minutes. Therefore, Traut's Reagent is usually used in excess amount and in amine free or low amine content buffers at nearly pH 8.5. Here, 100 fold molar excess of Traut's Reagent in phosphate buffer (pH: 8) was used for the thiolation of antiHer2 (2 hours at room temperature). (Scheme 3.7)



Scheme 3. 7 Synthesis of the thiolated antiHer2 (antiHER2-Dye-SH)

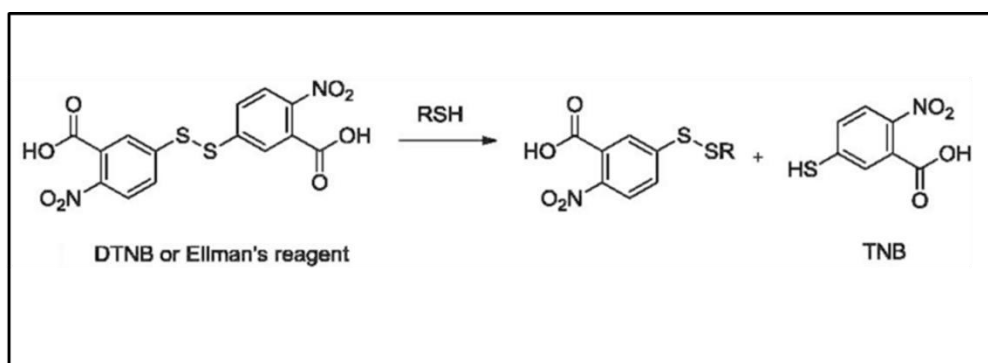
As seen in the reaction mechanism (Scheme 3.8), the reaction was carried out at pH 8 in order to enhance the nucleophilicity of primary amines on antiHER2, because pK_a values of primary amines are in the range of 9 to 10. Since majority of the primary amines are deprotonated at pH 8, their nucleophilic attack is facilitated at this pH and reaction efficiency is improved.



Scheme 3. 8 Synthesis mechanism of the thiolation of antiHer2 (antiHER2-Dye-SH).

Another important consideration when introducing sulfhydryl groups on proteins is avoiding the oxidation of sulfhydryl groups into the disulfide bridge. Since sulfhydryl groups are highly reactive, oxidation may take place in few hours or with the catalytic effect of divalent metal ions. In order to prevent metal catalyzed oxidation of thiol groups, a metal chelating agent ethylenediaminetetraacetic acid (EDTA) is used to chelate divalent metals ions in the environment. This procedure facilitates the reaction and the purification processes, eventually increases the yield.

For an efficient thiolation, at least 5-7 thiols per antibody are desired for a thiol-ene reaction. Amount of thiols can be quantified by Ellman's Assay which is a UV-Vis calorimetric test.[272] The detection mechanism of free thiols is based on the shift in the absorbance wavelength of 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) when interacted with the thiol groups in the medium. (Scheme 3.9).



Scheme 3. 9 Working mechanism of Ellman's Assay (Conversion of DTNB to TNB when interacted with thiol moieties)

Using the absorbance maxima at 412 nm of 2-nitro-5-thiobenzoic acid (TNB) and its extinction coefficient, amount of free thiols are calculated from the stoichiometric ratio between the thiols on antiHer2 and TNB.

$$A = \epsilon \times b \times c$$

$$0.003 = 14,150 \text{ M}^{-1}\text{cm}^{-1} \times 0.1 \text{ cm} \times c \quad (12)$$

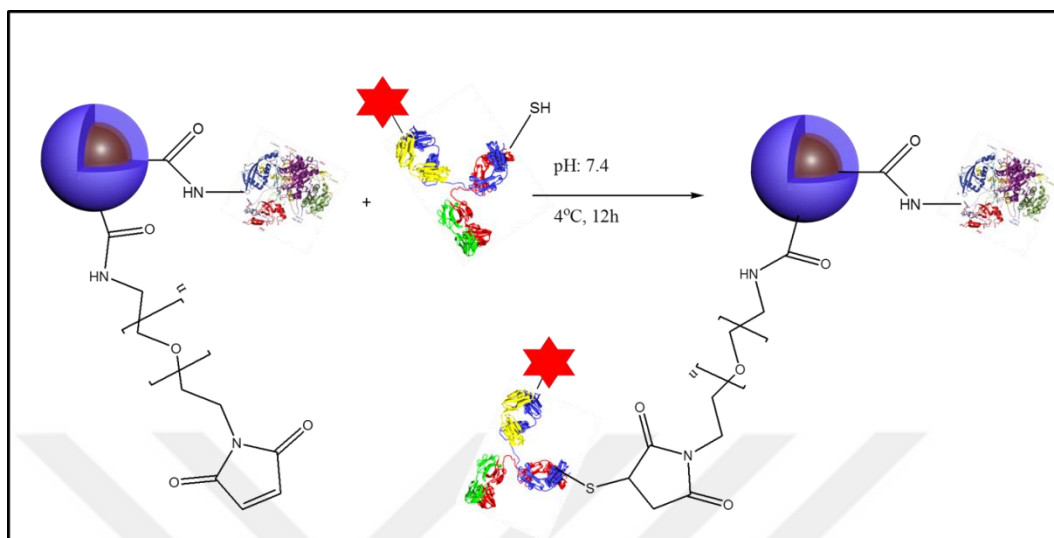
Using the equation (12), it was found that each antiHer2 has about 55 thiol (sulfhydryl) units. (Table 3.6) This number is sufficient to target maleimide units on SPION/AGO/MI.

Table 3. 6 Calculation of the number of thiol units on antiHer2-Dye by Ellman's Assay.

Sample	Amount of thiols (nmol)	Amount of Protein (nmol)	Number of sulfhydryls per protein
antiHer2-Dye-SH	35	0.64	~ 55

As a next step, thiole-ene chemistry was performed between antiHer2-Dye-SH and SPION/AGO2/MI (Scheme 3.10). Thiol-ene reaction, namely alkene hydrothiolation, is accepted as a special kind of “click reaction” in organic chemistry. This click reaction type is

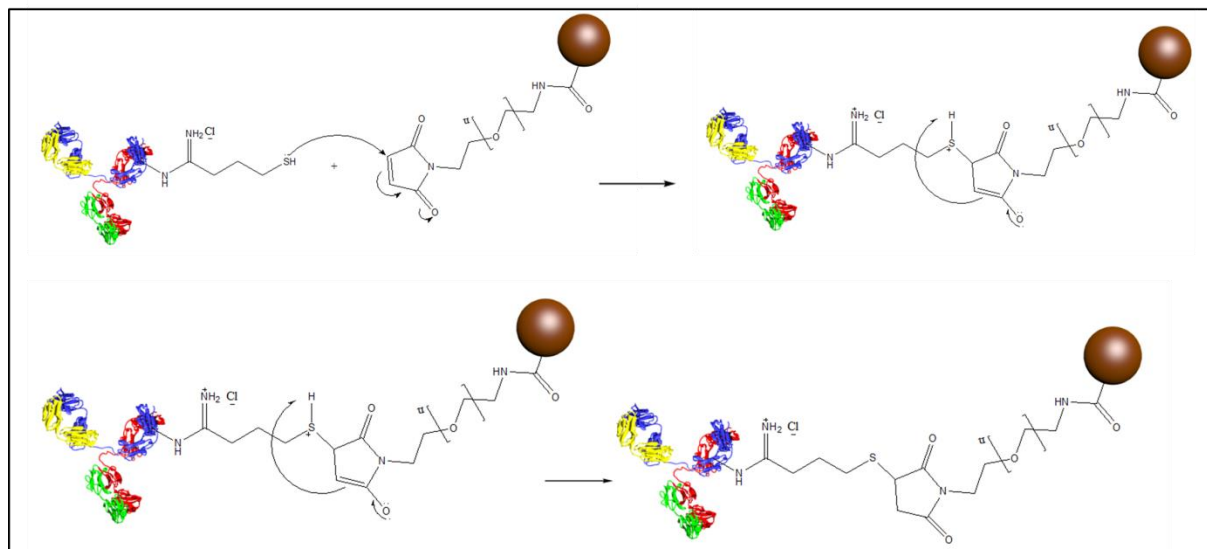
mostly used in bio-conjugation, because it is a very versatile process producing non-offensive byproducts and not sensitive to air or water.



Scheme 3. 10 Synthesis of the antiHer2-Dye conjugated SPIONs (SPION/AGO2/antiHer2-dye).

Reaction between the thiol and maleimide groups is a type of Micheal addition. The driving forces behind the reaction are the electron withdrawing effect of carbonyl groups and the ring strain on the maleimide moieties. A succinimidyl thioether product of SPION and antiHer2 was formed as the product. (Scheme 3.11) This reaction is highly efficient at pH 6.5 – 7.5, because this pH range provides high selectivity for the nucleophilic attack of thiols on proteins rather than nucleophilic attack of amines on the same protein. If pH is raised above 8, both thiols (pKa is around 10) and primary amines (pKa is around 9) may be deprotonated, and amines would be a better nucleophile and react with the maleimide groups.

During the thiol-maleimide reactions, EDTA was also added to the reaction media for the inactivation of metal impurities which catalyzes the oxidation of thiol groups to the disulfide bridge.



Scheme 3. 11 Synthesis mechanism of the thiol-maleimide conjugation between the MI active SPIONs and the thiol active antiHer2.

Attachment of the dye molecule on antiHer2 also provided a means to calculate the amount of antiHer2 conjugated to SPIONs. The concentration of DyLight 650[®] on the overall bio-conjugate (SPION/AGO2/antiHer2-dye) was calculated using the calibration curve created from the luminescence of the free dye (Figure 4A). Since it was previously figured out that, 0.8 dye molecules was attached per mole of antiHer2, the amount of antiHer2 on SPIONs was calculated as 19 $\mu\text{g/mL}$ (1.58 μg per mg of nanoparticles) (Table 3.4). As explained in the experimental section, this means around 24 antiHer2 proteins per nanoparticle.

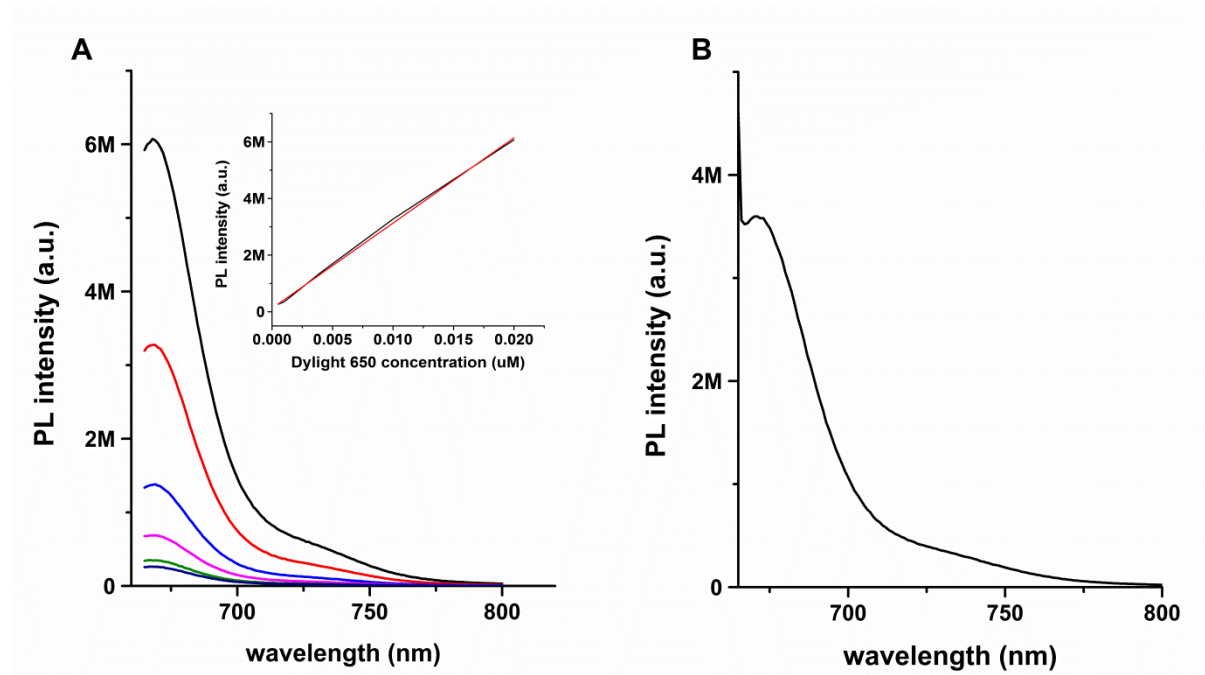


Figure 3. 7 Measurement of the antiHer2 concentration by photoluminescence (PL) spectroscopy. (A) PL spectra of Dylight 650[®] at different concentrations (2×10^{-2} , 1×10^{-2} , 4×10^{-3} , 2×10^{-3} , 1×10^{-3} , 5×10^{-4} μM , λ_{ex} : 655 nm, inset : Calibration curve of PL intensity vs concentration of DyLight 650[®] (B) PL spectrum of SPION/AGO2/antiHer2 (λ_{ex} : 655 nm, dilution factor : 20)

Table 3. 7 Calculation of the amount of antiHer2 on SPIONs using PL intensity of the dye.

Dylight [®] on SPIONs (nmol) ^a	Dylight [®] :antiHer2 (molar ratio) ^b	antiHer2 on SPIONs (nmol)	antiHer2 per mg of particle (μg)
0.114	0.8 :1	0.142	24

a Calculated by the calibration curve of reference Dylight 650

b Calculated by Beer Lambert equation ($\text{Abs}_{\text{protein}} = 280 \text{ nm}$, $\text{Abs}_{\text{Dylight}} = 655 \text{ nm}$)

When the conjugated amounts of dyes and proteins are calculated, one should not forget that, the numeric results are statistical. Each particle may have different amount of these.

For further confirmation of the protein conjugation on SPIONs, FTIR spectra of SPION/AGO2 and SPION/AGO2/antiHer2 were analyzed. (Figure 3.8) Proteins can be characterized through their typical IR bands in the mid IR region (amide A, B, I, II, III, IV).[273] In case of SPION/AGO2 and SPION/AGO2/antiHer2, resolving amide bands of proteins from the typical IR stretching and bending modes of PAA is difficult, therefore protein structure on SPIONs unfortunately was not fully characterized. However, bands at 1150 cm^{-1} in both spectra is most probably indicating the presence of the C-N stretching (Amide III band) of proteins.

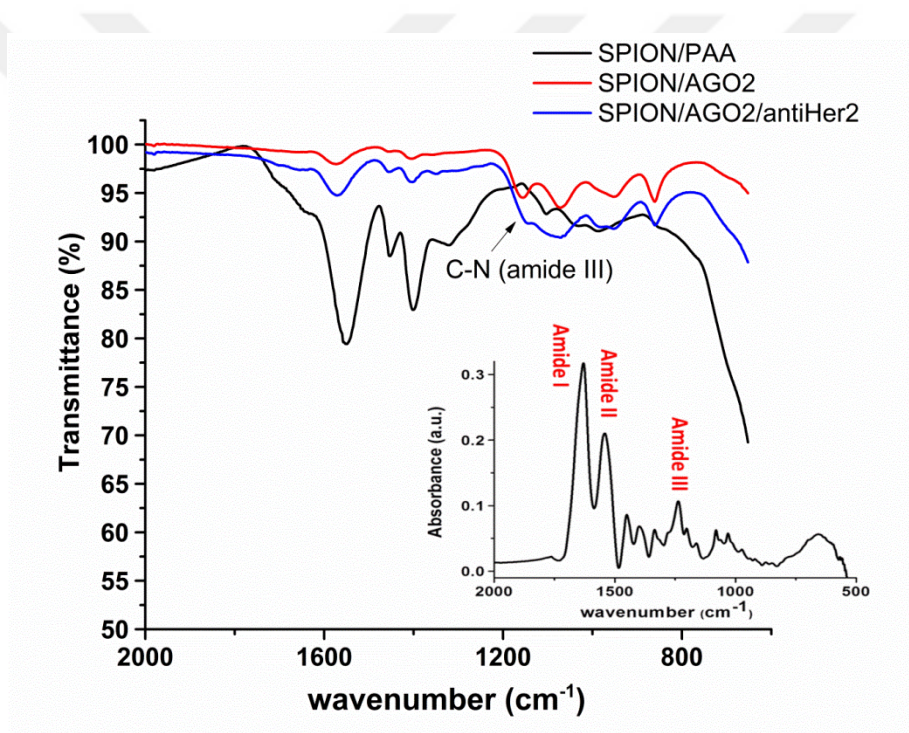


Figure 3. 8 FTIR spectra of SPION/PAA, SPION/AGO2 and SPION/AGO2/antiHer2. (inset: FTIR spectrum of gelatin showing characteristic amide I, II, II IR bands of a protein.)

3.4.5. Hydrodynamic Properties of the SPION-Protein Bio-conjugates

Hydrodynamic properties of bio-conjugates are crucial when they are used for therapeutic purposes, especially for the *in vivo* applications. Decrease in the colloidal stability after bio-conjugation would diminish their use in biological applications. Besides, as it was explained

in the introduction part, size of the particles influences the pharmacokinetics. Therefore, massive aggregation that would cause loss of colloidal stability or significant amount of aggregation increasing the size average over 150 nm is not allowed in this project.

Hydrodynamic size of SPIONs were measured by DLS after each conjugation step to monitor the change in the size and the size distribution (Table 3.8). As it is seen in the Figure 3.9 and 3.10, number average hydrodynamic size of SPION/PAA was increased from 14 to 20 nm after AGO2 was attached, while intensity average size distribution was dropped slightly from 122 to 115 nm. Change in zeta potential value from -19 meV to -23 meV designates the improvement in particle-particle repulsion. (Table 3.8)

Significant increase in the PDI and the drop in the zeta potential of SPION/AGO2 when maleimide units are introduced refer to the coagulation of nanoparticles due to the hydrophobicity of maleimide. (Figure 3.11, 3.12) Conjugation of antiHer2-Dye-SH recovers the colloidal stability of nanoparticles which can be observed with the decrease in PDI from 0.59 to 0.23 and increase in zeta potential from -21 to -24 meV which is due to the replacement of hydrophobic maleimide functional groups with hydrophilic antiHer2-Dye (Table 3.8).

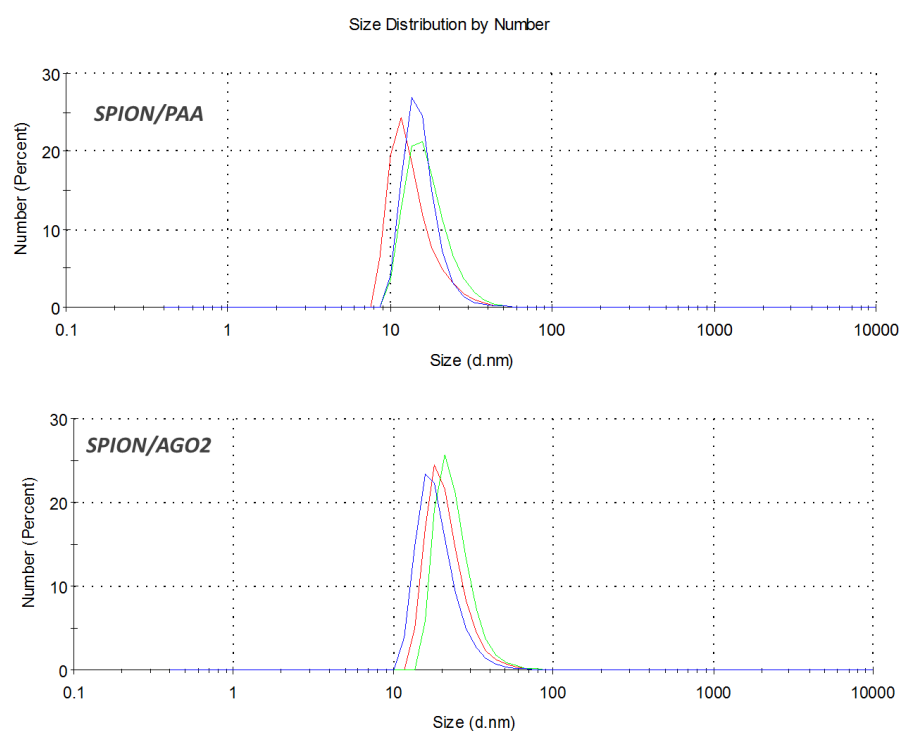


Figure 3. 9 Number average hydrodynamic size distribution of SPION/PAA and SPION/AGO2.

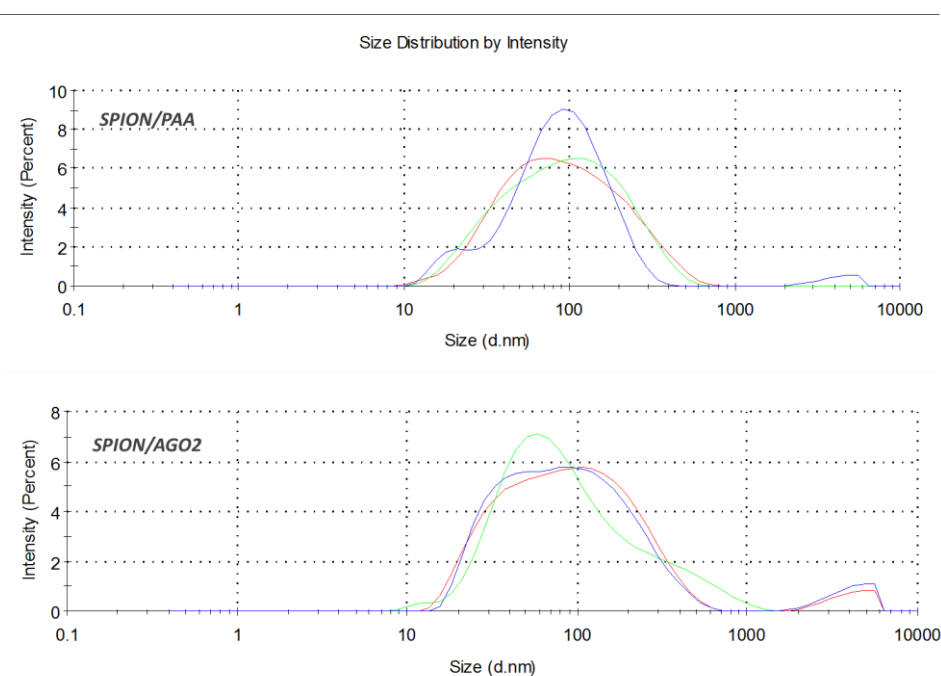


Figure 3. 10 Intensity average hydrodynamic size distribution of SPION/PAA and SPION/AGO2.

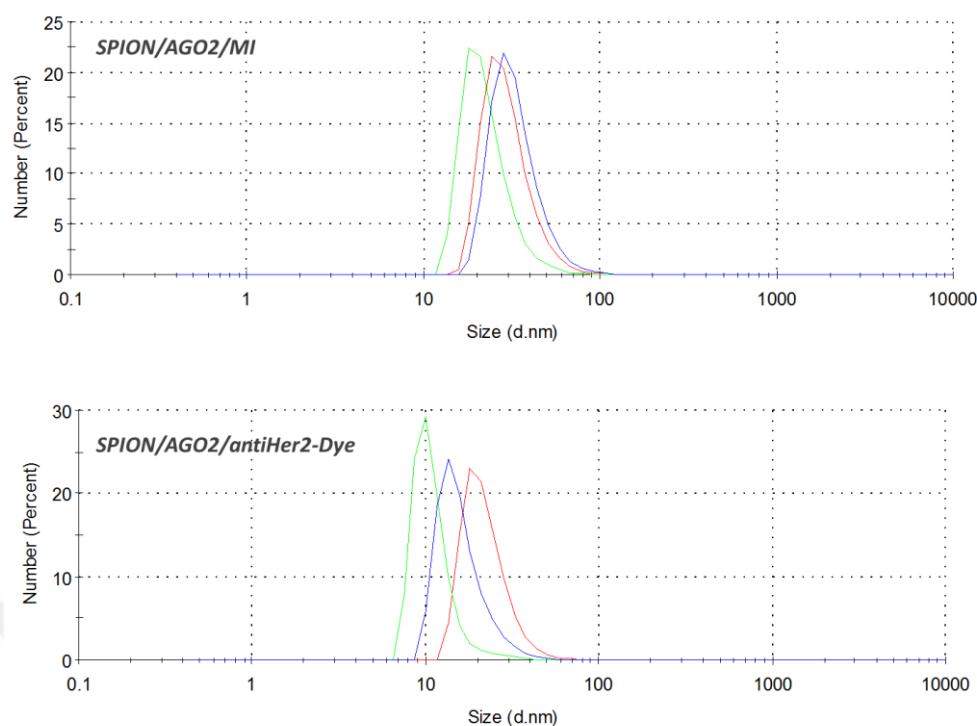


Figure 3. 11 Number average hydrodynamic size distribution of SPION/AGO/MI and SPION/AGO2/antiHer-dye.

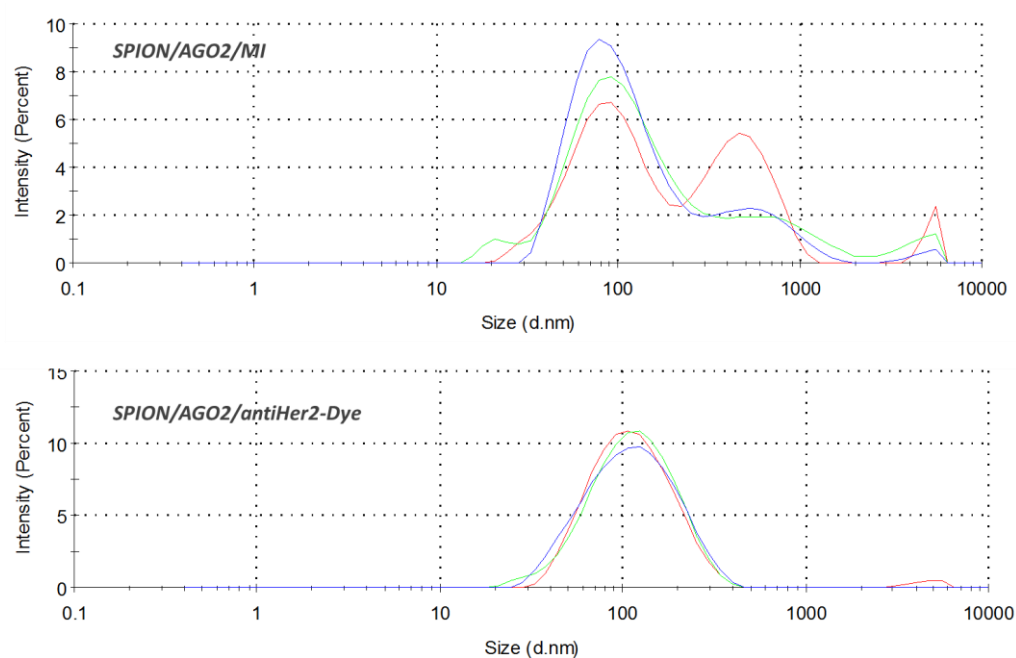


Figure 3. 12 Number average hydrodynamic size distribution of SPION/AGO/MI and SPION/AGO2/antiHer2-dye.

Table 3. 8 Hydrodynamic properties of SPION-protein bio-conjugates.

Sample	Num. average HDR ^a (nm)	Int. average HDR ^b (nm)	PDI	Zeta Potential ^c (mV)
SPION/PAA	14	122	0.40	-19
SPION/AGO2	20	115	0.43	-23
SPION/AGO2/MI	30	97 (57%), 487 (40%)	0.59	-21
SPION/AGO2/antiHer2-dye	49	123	0.23	-24
AGO2	100	460	0.40	-3.6
antiHer2-dye	233	279	0.90	-15

^a Number average size distribution^b Intensity average size distribution^c Hydrodynamic size and zeta potential measurements were taken in PBS buffer (pH:7.4)

Colloidal properties of antiher2-dye and AGO2 proteins were also measured to determine their hydrodynamic size and surface charge distributions (Table 3.8). Their size and PDI indicates that they are in the aggregated form. Since both antiHer2 and AGO2 have negative zeta potential values (Figure 3.13), conjugation with proteins did not alter the surface charge of the nanoparticles dramatically.

Here, the final bio-conjugate, SPION/AGO2/antiHer2-dye, is colloiddally stable and small enough (123 nm, int. av.) for *in vivo* use. Constructed nanoparticles are not too large to be cleared by RES or not too small to be subjected to a quick renal clearance. This final SPION construct is negatively charged with enhanced repulsive interactions (-24 meV). Since anionic charge is poorly reactive with the cellular compartments, they are expected to be much more biocompatible than the cationic gene delivery vehicles. Overall, designed and developed SPION bio-conjugates are colloiddally stable, small and negatively charged. These favorable hydrodynamic characteristics of nanoparticles are for favorable long blood circulation half life.

3.4.6. Miscellaneous Approaches for the Synthesis of SPION/AGO2/antiHer-dye

As it is mentioned before, several SPION compositions were tested along with SPION/PAA at the beginning of the study. These SPIONs differ in their coating hence surface chemistry. Therefore, different PEG linkers and conjugation chemistries were tested with each. Aminopropyltrimethoxysilane (APTMS), carboxyethylsilanetriol (CES) and dimercaptosuccinic acid (DMSA) coated SPIONs were synthesized and characterized for the conjugation of AGO2 and antiHer2-dye (Figure 3.13).

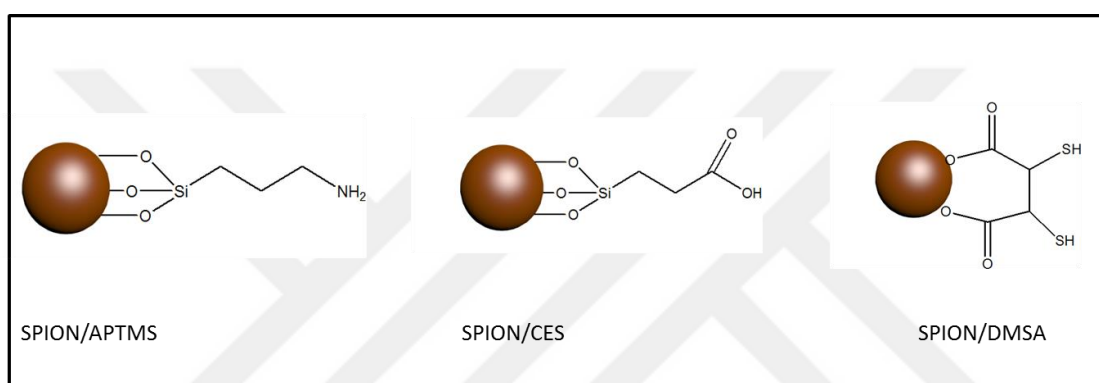
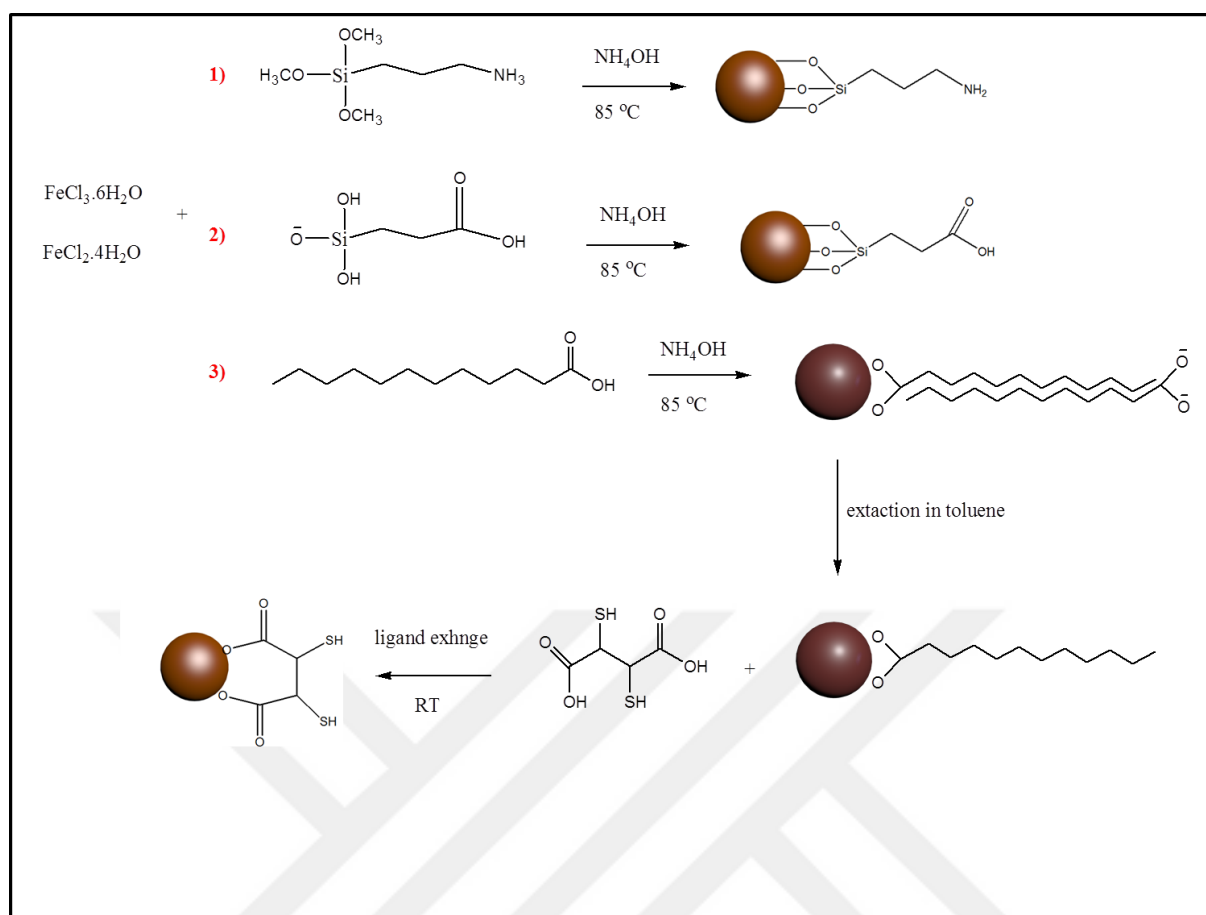


Figure 3. 13 SPIONs with different coating materials for the production of SPION-protein bio-conjugates.

Synthesis procedures of given SPIONs are given in the Scheme 3.12. SPION/APTMS and SPION/CES were synthesized using organofunctional alkoxy silane molecules APTMS and CES. (Scheme 3.12 1,2)



Scheme 3. 12 The synthesis of the SPION/APTMS (1), SPION/CES (2), SPION/DMSA (3)

SPION/DMSA was synthesized through the ligand exchange reaction on lauric acid coated SPIONs in toluene. (Scheme 3.12, 3) Reaction takes place by the replacement of lauric acid from the SPION crystal surface by DMSA.

Properties of these SPIONs are summarized in the Table 3.9. All of these SPIONs were colloiddally stable and small, hence were suitable for the project.

Table 3. 9 Some characteristic features of base SPIONs

Sample	Active Group	Organic Content (%)	Num.Av. HDR (nm)	Zeta Pot. (meV)
SPION/APTMS	Amine	15	15	+28
SPION/CES	Carbonyl	30	16	-36

SPION/DMSA	Sulfhydryl	~*	10	-22
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*Sample is not appropriate for thermogravimetric analysis because of the sulfhydryl units.

Although these functional SPIONs are promising scaffolds to generate successful and active SPION bio-conjugates, there were several synthetic problems during the process of making SPION-protein complex. In fact, these particles were abandoned due to the loss of colloidal stability and poor reproducibility during the conjugation summarized in Table 3.10.

Table 3. 10 Unsuccessful methods during the development of SPION/AGO2/antiHer2-dye

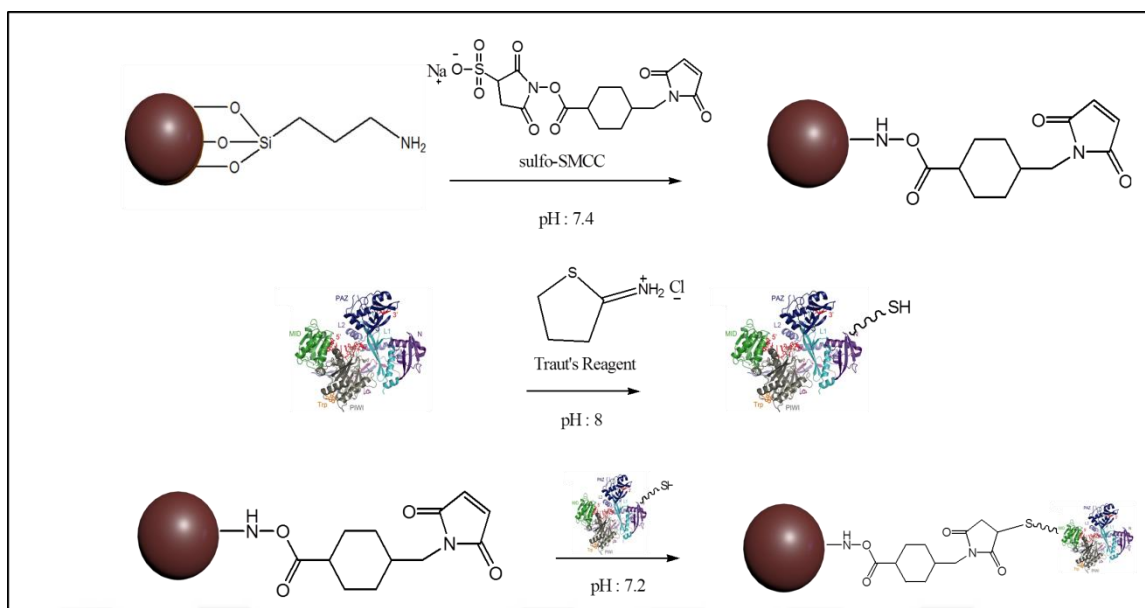
Base Particle	AGO2 coupling method	AntiHer2 –dye coupling method	Efficiency of the method	Problem
SPION/APTMS	Thiol-ene conjugation between the thiol active AGO2 and the maleimide active SPIONs (Scheme 3.13)	-	AGO2 conjugation was unsuccessful	Precipitation of SPIONs after conjugation with sulfo-SMCC ^a
SPION/APTMS	EDC/NHS conjugation between the amines of SPIONs and the carbonyls of AGO2.	Disulfide bridging through thiol modified SPIONs and sulfo LC-SPDP ^b modified antiHer2-dye (Scheme 3.14)	-AGO2 conjugation was successful , -antiHer2-dye conjugation was unsuccessful	Quenching of far red dye after conjugation
SPION/CES/NTA	Ni-Histidine coordination between the Ni coordinated SPIONs and polyhistidine (6) tagged AGO2.	-	AGO2 conjugation was unsuccessful	Reproducibility problem due to the precipitation of SPION/CES at pH 6

	(Scheme 3.15)			
SPION/DMSA	Amidation between the NHS ester active SPIONs and amines of AGO2. (Scheme 3.16)	-	AGO2 conjugation was unsuccessful	Precipitation of SPIONs after conjugation with sulfo-SMCC.

^aSulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate.

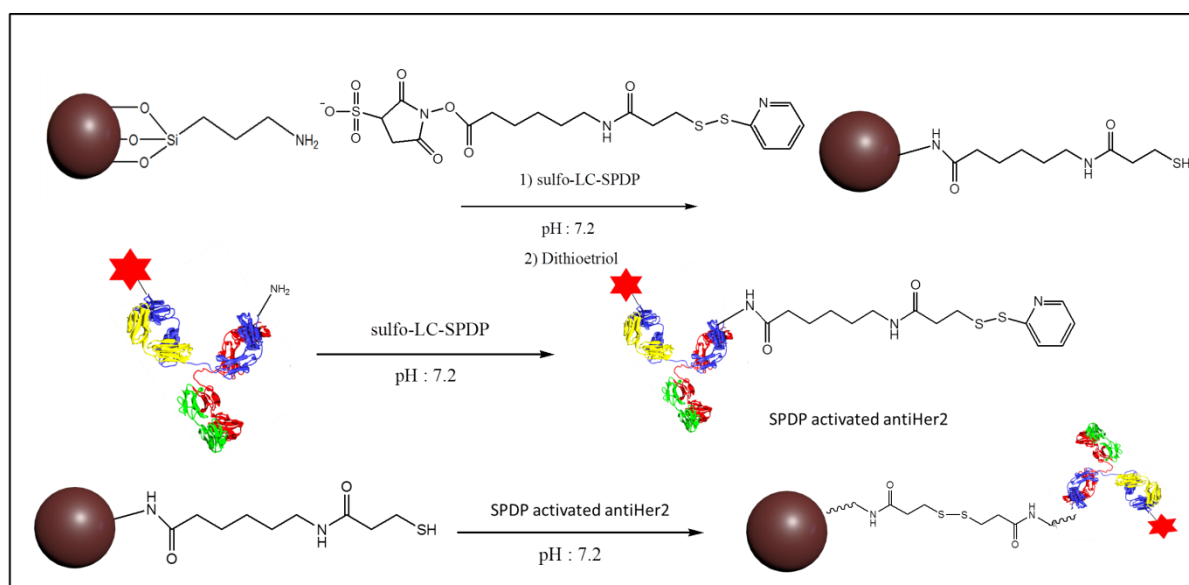
^bSulfosuccinimidyl 6-(3'-(2-pyridyldithio)propionamido)hexanoate

SPION/APTMS is a widely used SPION composition that was tried in this study as well. The overall AGO2 conjugation to these particles is shown in Scheme 3.13 which involves the use of a sulfo-SMCC which would bind to SPION via amide bond and render particles MI functional which would react with thiolated AGO2 in the second step. This is one of the common bioconjugation chemistries and a typical reagent. One of the reasons of using sulfo-SMCC is to attach AGO2 to surface via a short linker (8.1Å) to provide some free space to each AGO2 to attain their favored conformation and not to hinder the approach of another one during the synthesis. However, the conjugation of AGO2 to SPION/APTMS was problematic because of the precipitation of SPIONs when conjugated with sulfo-SMCC even at low stoichiometric ratios due to the hydrophobic nature of the sulfo-SMCC (Scheme 3.13). Therefore, this method was not successful.



Scheme 3. 13 Synthesis of SPION/AGO2 from SPION/APTMS by thiol-ene conjugation.

Since SPION/APTMS was precipitated after the reaction with the crosslinker sulfo-SMCC, a new crosslinker was preferred to react with SPION/APTMS which is sulfo-LC-SPDP. This reagent also renders particles hydrophobic during the synthesis but eventually provides thiol functionality upon reduction of dithiol bond with dithioetriol (DTT). This produced thiol functionalized SPION/APTMS. Parallel to this, antiHer2 was also conjugated with sulfo-LC-SPDP, but it was not reduced by DTT, instead it was reacted with thiolated SPION/APTMS to provide antiHer2 conjugation via a disulfide bond (SPION/S-S/antiHer2-dye) (Figure 3.14).



Scheme 3. 14 Conjugation of antiHer2-dye to SPIONs by thiol oxidation method.(SPION/S-S/antiHer2-dye)

However, this method produced particles with bimodal charge distribution (Table 3.11) but more importantly it caused total quenching of the dye conjugated to antiHer2 (Figure 3.14). Which parameter of the reaction produces such result is not clear but this method was abandoned, as well.

Table 3. 11 Hydrodynamic properties of SPION/S-S/antiHer2-dye

Sample	Int. av. HDR (nm)	Num. av. HDR (nm)	Zeta Pot.* (mV)	PDI
SPION/PAA	130	42	+30.5	0.28
SPION/S-S/antiHer2	157	17	+37 (66%) +14 (33%)	0.38

*Measurements were taken in HEPES buffer pH : 7.4.

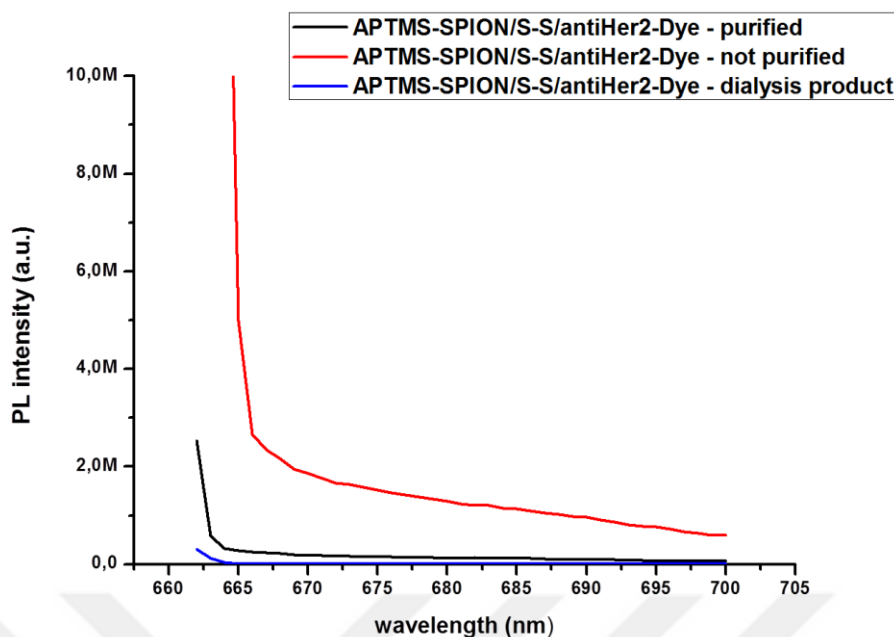
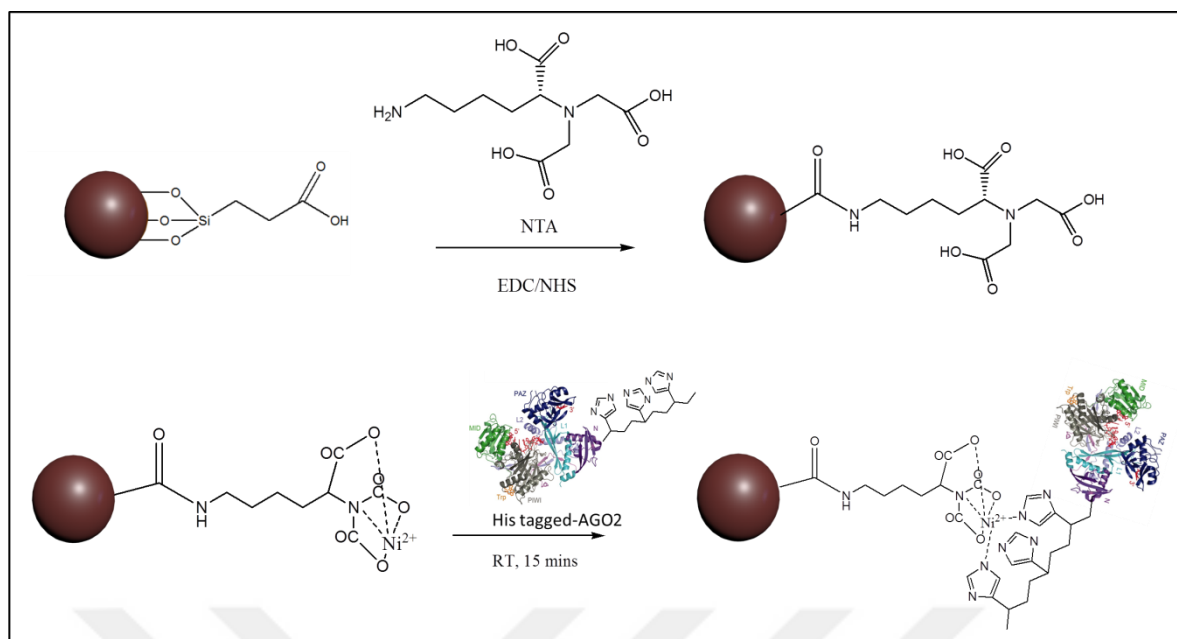


Figure 3.14 PL spectrum of pure, impure SPION/S-S/antiHer2 and dialysis product.

Another significant approach in the synthesis of SPION-AGO2 bio-conjugate is attachment of SPIONs with nitriloacetic acid (NTA), than tried to be conjugated with the six histidine (his tag) terminus of the AGO2 via Ni^{2+} complexation, which is a known coordination chemistry in the literature. This is a highly efficient method used in affinity chromatography. Since 6 histidine moieties (his tag) have affinity to Ni^{2+} , his tagged proteins are purified using Ni^{2+} chelating columns. Inspired by this application, we designed a facile synthetic method to create SPION/AGO2 complex. (Scheme 3.15) This method provides a gentle reaction condition for AGO2. In a sense, it would be possible to create a strong SPION/AGO2 complex only by incubating AGO2 with SPION/NTA. For this purpose SPION/CES was produced. However, the method lacks reproducibility and colloidal stability in buffers. Hence, this approach was abandoned, as well.



Another simple approach to produce SPION/AGO2 is linking AGO2 and NHS active SPIONs in a single step reaction. This time amine functionality of AGO2 is used and AGO2 thiolation is needed, leaving AGO2 more natural. Since it may seem that the use of NHS activated SPIONs are feasibly attached by AGO2 unless harm its structure of functionality, this method is preferred using DMSA coated SPIONs. Again, the popular linker sulfo-SMCC was planned as the linker, so a thiol functional SPION was synthesized with DMSA coating. SPION/DMSA reacted with sulfo-SMCC producing an NHS functional SPION. However, the nanoparticles precipitated again once reacted with sulfo-SMCC at different stoichiometry. Therefore, this strategy was not followed up either.

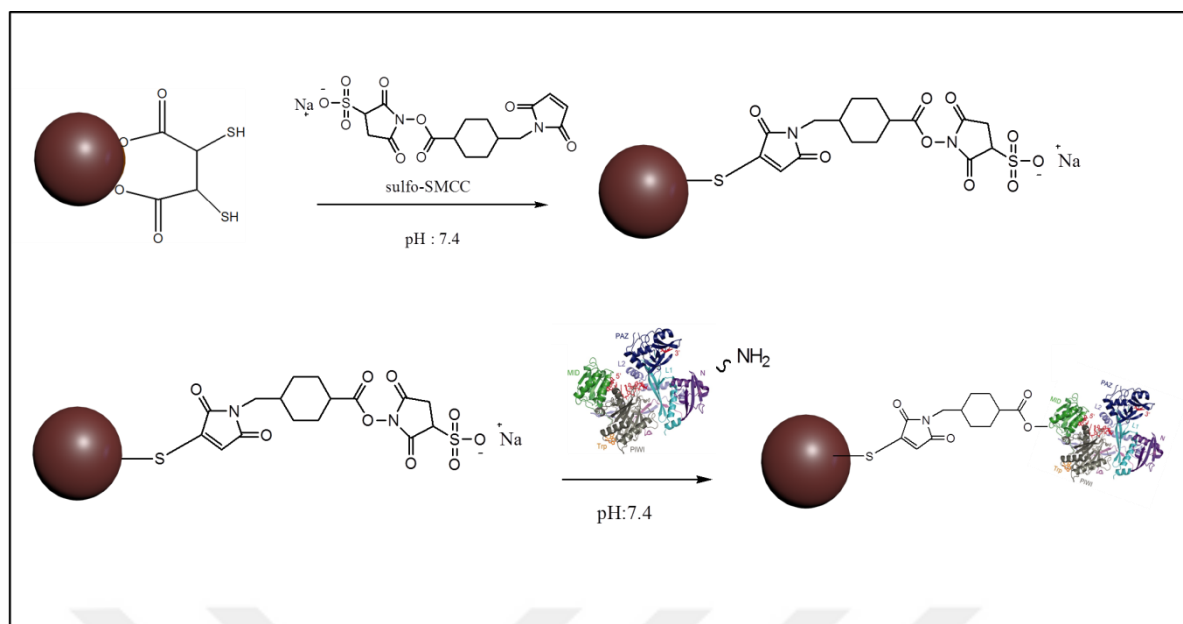


Figure 3. 16 Synthesis of SPION/AGO2 starting from SPION/DMSA.

The experience gained from these trials show that colloidal stability is very sensitive to the synthesis conditions and the chemistry of the activators and linkers, especially with these small molecule coated SPIONs. Therefore SPION/PAA was used as the core particle which is completely stable at a large pH range (4-8) and highly magneto-responsive. All applied synthetic procedures show that the stability of nanoparticles is an essential factor which has to be carefully controlled during the bio-conjugation steps. Since there exist numerous linkers and conjugation methods to generate nanoparticle-protein complexes, a smart design that is compatible with the scope of an ultimate bio-conjugate complex can be obtained by the detailed and optimized experimental work as described in this thesis.

3.4.7. *In vitro* and *in vivo* biocompatibility of SPION/AGO2/antiHer2-dye bio-conjugates

Since the ultimate goal is the application of the developed SPION bio-conjugates to use them as therapeutic agents eventually for cancer therapy, the first critical test is the biocompatibility determination. *In vitro* cytotoxicity assessment was done by a special cell viability assay

MTT, which examines the cellular activity. The working principle of this assay is based on the reduction of yellow tetrazolium MTT to its purple formazan product by the mitochondrial enzymes in the cellular medium. [274] MTT data show the cell proliferation rate of the breast cancer cells, SKBR3 and MDA-MB-453, when exposed to SPION bio-conjugates. (Figure 3.17 a) Particles with only antiHer2 (SPION/antiHer2-dye), with both AGO2 and antiHer2 (SPION/AGO2/antiHer2-dye) and their complex with miR376b (SPION/antiHer2-dye/miR376b, SPION/AGO2/antiHer2-dye/miR376b) were tested. They all showed good biocompatibility in 48 h incubation period when given to cells at 0.15 mg/mL dose. Statistical analysis of the results indicates a completely safe bio-conjugate system for *in vitro* consideration. Toxicity of the final SPION/AGO2/antiHer2-dye structure was also tested *in vivo* using nude mice along with the core particle, SPION/PAA. Blood biochemistry test performed after 10 and 40 days after injection of nanoparticles at the dose of 0.15 mg/mL confirm the biocompatibility of SPION/AGO2/antiHer2-dye (SP-AH). The levels of the various parameters in the blood plasma were not affected after the IV injection of either nanoparticle (Figure 3.17 b). Hence, bio-conjugation of SPION/PAA with antiHer2 and AGO2 do not negatively influence its biocompatibility.

These results indicate that developed SPION-protein bio-conjugates are non-toxic at the studied dose and its theranostic potential can be investigated further. Such superb biocompatibility originates from the critical design which brought together a relatively non-toxic iron oxide crystal, biologically reliable and anionic coating material PAA, non-cationic AGO2, biocompatible antiHer2 as a targeting ligand, and the synthetic strategies which produced such combination in small size regime and in a colloiddally stable form. Here, it must be remembered that polycationic gene delivery agents are not that much biocompatible even all the other parameters are preserved.[240]

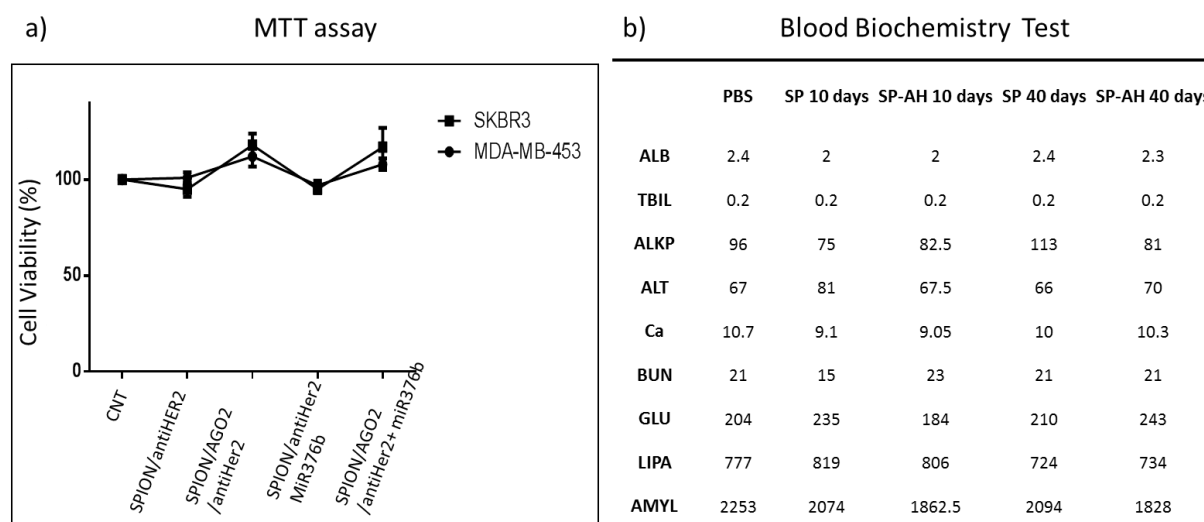


Figure 3. 17 Biocompatibility analysis of SPION-protein bio-conjugates. a) MTT assay of SPION-protein bio-conjugated with and without mir376b at the dose 0.15 mg nanoparticles after 48 h incubation. b) Blood biochemistry test of 0.15 mg of SPION/AGO2/antiHer2 bio-conjugates on nude mice with 48 h intravenous post-injection. (SP: SPION/PAA, SP-AH : SPION/AGO2/antiHer2-dye) (*Data generated by Muhammed Koçak, Yunus Akkoç, Dr. Devrim Gözüaçık*)

3.4.8. *In vitro* and *in vivo* targeting efficiency of SPION/AGO2/antiHer2-dye bio-conjugates

The specific binding capability of an antibody to a receptor on the cell surface takes an advantage of targeting selective epitopes on the tumor side. Therefore, by using antibody technology therapeutic payloads can be delivered to the disease in a more selective manner. This is the new generation drug technology because chemotherapeutics can be internalized at higher concentration at the intended area and their effect can be improved.

In vitro tumor targeting capability of antiHER2 tagged SPION/AGO2 nanoparticles was tested on breast cancer cell lines of SKBR3 and MDA-MB-453 which overexpress Her2 receptor and MCF cell line which has a low level of Her2 receptor. Targeting efficiency of

nanoparticles was analyzed by detecting the far red emission of the dye conjugated to the antiHer2 conjugated to SPION/AGO2. After incubation of these cells with 0.15 mg/mL nanoparticles for 48 hours, the fluorescence signal from the cells was determined under the fluorescence microscopy. As seen in the fluorescence microscopy images (Figure 3.17), Her2 positive SKBR3 and MDA-MB-453 cells have a strong red emission while Her2 negative MCF 7 cells do not give any fluorescence signal when exposed to the nanoparticles.

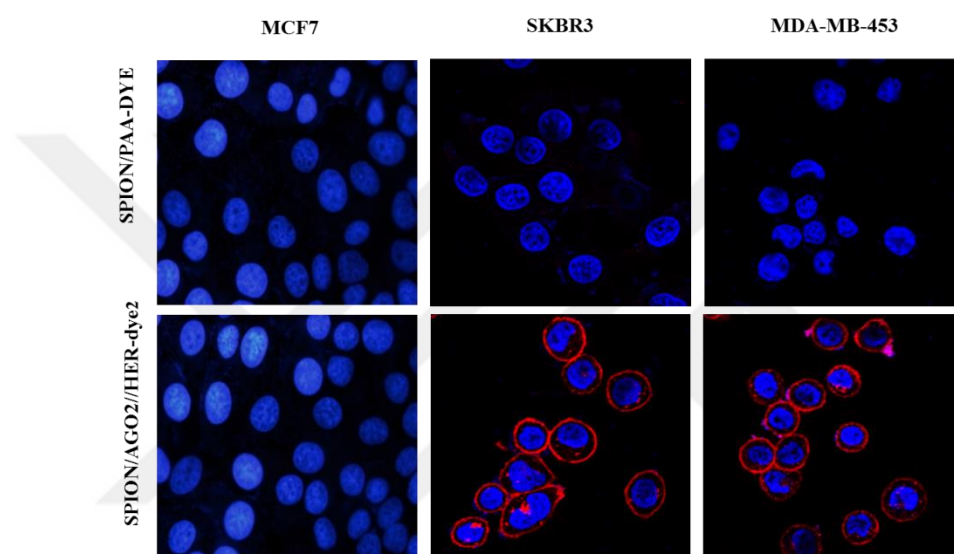


Figure 3. 18 Fluorescence microscopy images of MCF7, SKBR and MDA-MB-53 breast cancer cell lines exposed to 0.15 mg/mL SPION/AGO2/antiHer2-dye and SPION/PAA-dye for 48 h. (*Data belong to Muhammed Koçak*)

As a control, SPION/PAA was also tagged with the same far red dye Cy5 and incubated with these cells at the same concentration and conditions. Any fluorescence signal was detected from the cells. This supports that anionic nature of the SPION/PAA does not provide a dramatic passive uptake and anti-HER2 tag is successfully delivering particles to target cells with overexpressed Her2 receptor.

In vivo targeting efficiency of the nanoparticles was also confirmed using nude mice models with SKBR3 and MDA-MB-453 tumors. In order to demonstrate selective uptake by tumor tissues, red fluorescence of SPION/AGO2/antiHer2-dye was monitored using an *in vivo* imaging system, IVIS. Bright and red epi-luminescence signals from both SKBR3 and MDA-MB-453 tumor tissues removed from animals after 48 h after intravenous injection of the nanoparticles (Figure 3.19). At least as important as this, lacks of an optical signal from the healthy organs suggest that the majority of the nanoparticles were accumulated selectively on the tumor sides as desired.

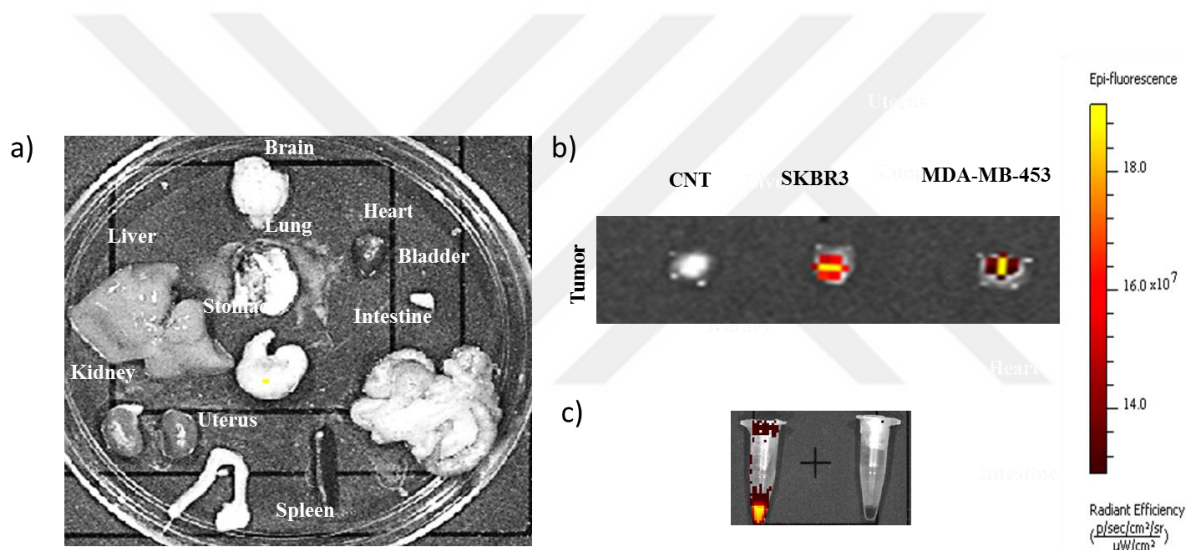


Figure 3. 19 *In vivo* fluorescence imaging of the organs taken from model nude mice using IVIS. 0.15 mg of SPION/AGO2/antiHer2-dye particles were injected intravenously to the nude mice and organs were harvested 48 h after injection. Fluorescence images of a) healthy and b) tumor tissues (SKBR3, MDA-MB-453: tumors delivered with the nanoparticles and CNT : tumors not delivered with the nanoparticles) c) Nanoparticles suspensions of SPION/AGO2/Her2-Dye (left) and SPION/PAA-dye (right) (**Data generated by Muhammed Koçak and Yunus Akkoç**)

In vitro and *in vivo* experiments show that developed nanoparticles have high affinity to the Her2 overexpressed breast cancer cells and show perfect targeting capability which can be used for smart delivery of miRNA therapeutics along with minimal uptake by the health organs. This is an extremely promising result.

3.4.9. *In vitro* miRNA delivery efficiency of SPION/AGO2/antiHer2-dye bio-conjugates

The most significant task of designed SPION bio-conjugates is to deliver therapeutic miRNAs onto the intended side successfully. In order to demonstrate their gene delivery capability, model autophagy related miRNAs and miR376b were loaded on SPION/antiHer2-dye (SP-H : negative control) and SPION/AGO2/antiHer2-dye (SP-AH: positive control), then they were incubated with SKBR3 and MDA-MB-453 breast cancer cells. The interaction mechanism of miRNAs with AGO2 protein is not clear like in case of the electrostatic interaction between a polycationic carrier and polyanionic gene. However, it is estimated that chemical conditions of the intracellular medium facilitates the interaction of miRNA and AGO2 by constructing two sided chelation through the cations between the miRNA and AGO2. This hypothesis is suggested by the *in vitro* loading conditions of AGO2 loading (Dr. Gozüaçık). Complexation studies of blank AGO2 or SPION/AGO2 with mimic miRNAs were carried out in the amine containing buffer solution with Mg^{2+} ions. (See 3.2.15) This complexation condition designates a need for a cationic bridge to interact with both anionic AGO2 and anionic miRNA and complex them together.

The delivery efficiency of the concerned miRNAs was comparatively tested with the miRNA and mRNA level. Here, only the changed miRNA levels are briefly discussed. Increase in the native miR376b level is the major confirmation for an efficient miR376b mimic delivery to the target breast cancer cells. The transcription fold changes of native miR376b were quantified by taq-man Q-PCR after internalization of the nanoparticles (48 h exposure).

(Figure 3.20) There is a very poor increase in the relative change of native miR376b level of SKBR3 cells when they were internalized by final complex containing AGO2. Hence, the increase in the level of the miR376b transcription in SKBR3 cells which were internalized with SPIONs as negative control is almost same in case of the final AGO2 containing SPIONs. This shows that efficiency of delivery or the activity of miRNAs are low in SKBR3 cells. However, the related fold change of miR376B is significantly increased (around 20 fold) in MDA-MB-453 cells after the uptake of final nanoparticle complex. Negative control, SPIONs without AGO2, do not show any remarkable increase in the transcription fold change of miR376b in MDA-MB-453. Relative transcription results of miR376b clearly confirms that, SPION bio-conjugates are able to load and deliver their payload, miR376b mimics, into the MDA-MB-453 cells in targeted manner. The difference in their effect on SKBR3 and MD-MB-376b is most probably arisen from the different internalization degree of the nanoparticles which may be the result if dissimilarity of the Her2 expression.

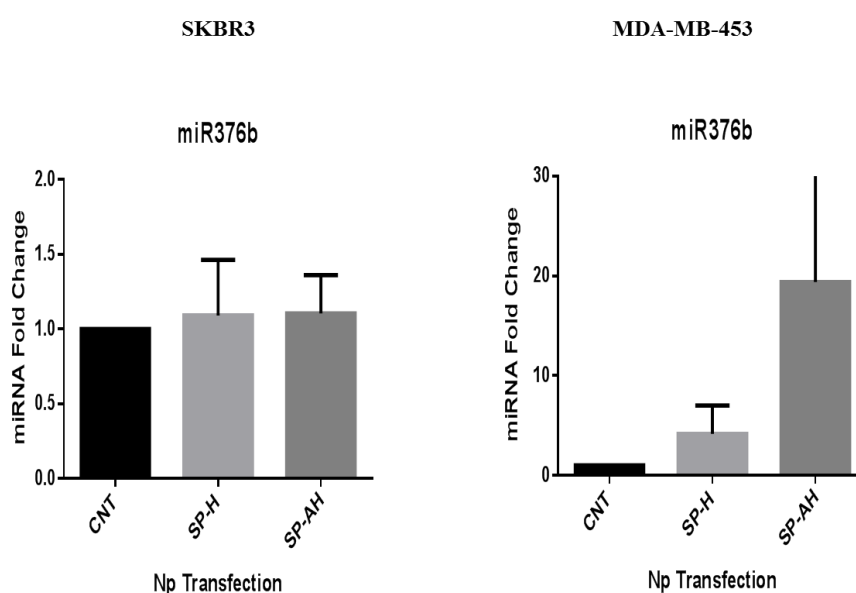


Figure 3. 20 Bar charts showing miRNA fold change when transfected with SPION/antiHer2-dye (SP-H) and SPION/AGO2/antiHer2-dye (SP-AH) into SKBR3 and MDA-MB-453 cells after 48 h exposure time. miR376b expression was analyzed using tag-man QPCR . The fold

change values were calculated by normalizing against U6 small nuclear 1 (RNU6-1). **(Data generated by Muhammed Koçak)**

3.5. Conclusions

First time in the literature, AGO2 is conjugated to a particle to generate a miRNA transfection vector which is highly biocompatible unlike typical cationic gene delivery vehicles. Through a very detailed design of components, conjugation sequence and conditions, small, stable SPION/AGO nanoparticles tagged with antiHER2-dye is developed again for the first time in the literature providing efficient miRNA binding and selective targeting of the Her2 overexpressed tumor cells and breast tumors.

In a more general sense, we have designed and developed multifunctional SPION-protein complexes to deliver therapeutic miRNAs into the breast cancer tumors in a most efficient way. In this context, we developed a novel and fundamental SPION based theranostic system which excludes the toxic effect of traditional polycationic gene carriers and delivers the miRNAs selectively tumors and can be optically and magnetically trackable.

Designed nanoparticles are synthetic and sophisticated analog of the RISC associated protein AGO2 which complexes miRNAs and carry them both inside and outside of the cellular media. Using AGO2 protein as miRNA carrying element in the theranostic SPIONs enables us to create polyanionic and biocompatible gene carrier model for the first time. Current gene delivery technology suffers from the toxicity of poly-cationic gene delivery components. When this major drawback is taken into account, development of the protein associated nanoparticles is a novel and promising approach in miRNA delivery.

SPIONs gain the molecular targeting capability by antiHer2 protein which selectively endocytosed by Her2 receptors. Since Her2 receptor expression is amplified in the 15-30 % of the breast cancer, Her2 is an excellent therapeutic target for breast cancer research. Therefore,

we proposed that the miRNA delivery efficiency to the concerned breast cancer is severely improved when associated with selective Her2 targeting. Imaging capability is a crucial characteristic of the new generation therapeutics. This modality allows tracking the therapeutic system in the cell or in the body tissues and ensures the therapeutic-tissue interaction. For this purpose, we attached fluorescent molecule on the SPIONs to track them under fluorescence microscopy. Iron oxide core is preferred for its bio-compatibility, ease of preparation and more importantly for their hyperthermia capability as an adjuvant therapy.

AGO2 and fluorescent antiHer2 conjugated SPIONs were carefully designed by taking the advantage of the bio-conjugation techniques. The ultimate goal of the bio-conjugation strategies is to produce an optimum composition incorporating the distinctive functionalities of each building block in a most perfect and practical manner. In this context, the process of creating SPION-protein bio-conjugates was carried out by applying some key spatial arrangements: AGO2 was directly attached to keep the miRNA payloads from the enzymatic media. antiHER2 was linked with a spacer to facilitate its match with the Her2. For this purpose, SPION-AGO2 was constructed using simple carbodimide crosslinker approach while antiHer2 was linked by highly specific thiol-maleimide chemistry. Synthesized nanoparticles are proved to be small enough (49 nm) to escape from the RES, colloiddally stable in the pH range 4-8 (-24 meV) designating the ability to circulate in the blood vessel without coagulation and brightly red fluorescent which can be tracked under fluorescence monitoring. Hence the amount of active proteins AGO2 (around 124 AGO2 per nanoparticle) and antiHer2-dye (around 24 antiHer2 per nanoparticle) on SPIONs were detected before the test of conjugate activity in its intended applications. *In vitro* MTT assay and *in vivo* blood biochemistry analysis are in confirmation with their envisioned bio-compatibility. They show that nanoparticles are almost non-toxic to the concerned breast cancer cells SKBR3, MDA-MB-453 and nude mice models at the dose of 10 mg Fe per kg and 24 h time exposure.

Fluorescence microscopy and IVIS spectrum studies demonstrate the highly selective internalization of final SPION-protein complex into the Her2 overexpressed breast cancer cells SKBR, MDA-MB-453 and their tumor models on nude mice. Finally, SPION-protein complex achieves a successful transfection of the miR376b mimics especially to the MDA-MB-453 cells. The transfection efficiency was confirmed by the increase in the level of native miR376b transcription. Entirely, developed ultrasmall, colloidal stable SPION-protein bi-conjugates are highly promising in targeted miRNA delivery.



CHAPTER 4

4. TARGETING BREAST CANCER WITH BISPECIFIC, ANTIBODY TAGGED SPIONS

4.1. Introduction

Various cellular dysfunctions in the cellular activities such as apoptosis, angiogenesis, proliferation, motility are responsible for the transformation of somatic epithelial cells to tumor which are deregulated through the change in genome.[275, 276] This leads into the formation of heterogeneous cellular subsections in cancerous tissues. Several models were proposed to explain cellular heterogeneity in tumors: *Clonal evolution model* focuses on mutations during carcinogenesis by demonstrating the interaction of lots of sub-clones in solid tumors.[277] *Cancer stem cell model* states that tumor cells are organized hierarchically and gain stem cell like properties such as self-renewal ability or differentiation.[278] Overall, genetic effects which generate oncogenes and the cell of the origin are the major contributors to the diversity of cancer subtypes.[279, 280] In case of breast cancer, around a thousand oncogenes which are effective on abnormal cell homeostasis are reported as.[281, 282] This makes breast cancer significantly a heterogeneous disease with different phenotypes and expression abnormalities.[283] Molecular subtypes of breast carcinomas were identified using DNA microarrays and grouped as follows : Luminal A, Luminal B, HER2 overexpressed, Basal, Normal.[284, 285] Given subtypes have different expression levels of protein biomarkers of estrogen (ER), progesterone (PR), human epidermal growth receptor 2 (HER2) and epidermal growth factor receptor (EGFR or HER1).[286, 287] Variance of these receptors on breast cancer results in different responses to therapeutics and eventually different clinical outcomes. [288, 289] Therefore biological heterogeneity in breast cancer cells has to be taken into account when *in vivo* and clinical studies are performed based on the *in vitro* results.

Breast cancer biology is studied extensively in the literature using numerous cancer cell lines and new bioengineered materials.

Nano-carriers which target the specific receptors (ER, PR, HER2, EGFR) on breast carcinomas are abundant. Subik *et al* harvested seventeen breast cancer types and determined their molecular subtypes with immunohistochemical analysis (IHC) [290]. The expression levels of receptors on breast cancer subtypes are given in the Table 4.1. Among the cell lines which are widely used in breast cancer research MCF 7 is classified as Luminal A, BT 474 are Luminal B, MDA MB 435 and SKBR3 are HER2 overexpressed and MDA MB 468 is classified in basal subtype.

Table 4. 1 Molecular classification of breast cancer subtypes. [290]

Subtype	ER and/or PR	HER2	EGFR
Luminal A	+	-	-
Luminal B	+	+	-
HER2	-	+	-
Basal like	-	-	+

Role of HER2 and EGFR in Breast Cancer Therapy

EGFR (HER1) and HER2 are both transmembrane receptor proteins having extracellular ligand binding and intracellular tyrosine kinase receptor domains with ligand binding activity in various mechanisms such as proliferation, growth, signaling, adhesion, motility and angiogenesis.[291, 292] Since HER2 and EGFR regulate significant signaling transductions, dysregulation of these glycoproteins can promote tumor progress.[293] EGFR and HER2 receptors are significant targets in breast cancer therapy due to the following hallmarks :

- Level of HER2 and EGFR proteins is directly related to carcinogenesis.[294]

- Overexpression of HER2 and EGFR is relatively high in tumor tissues compared to healthy tissues. [295]
- HER2 overexpression comprises both primary and metastatic tumor phase which makes HER2 an effective target along the carcinogenesis. [295]

HER2 inhibitors are favorable therapeutics against breast carcinoma. Herceptin[®] is the first drug which is a humanized monoclonal antibody, antiHER2. The mechanism of Herceptin activity is not fully clear but it is considered that this drug has both cytostatic (slows the cell growth) and cytotoxic (destroys the cell integrity) effects inside the cell and induces antibody dependent cellular toxicity through the activation of natural killer cells (NKs). [295]

EGFR receptor is generally overexpressed in triple negative breast cancer and inflammatory breast cancer which results in large tumor size and poor differentiation.[293] Several therapies targeting EGFR receptor are developed and Cetuximab[®] is one of the used commercialized chimeric monoclonal antibody which binds EGFR with high affinity. Cetuximab prevents cell proliferation, angiogenesis and metastasis through the activation of apoptotic pathways. [291]

Herceptin and Cetuximab modified nanocarriers for breast cancer

Since both HER2 and EGFR are significant biomarkers in breast cancer, antiHER2 (Herceptin) and antiEGFR (Cetuximab) are widely used in the 1) antibody treatments [296, 297], 2) synergetic therapies with chemotherapeutics [298, 299] or 3) targeting to the tumor tissue.[300, 301] Because of the complexity of tumor biology, anticancer efficacy of therapeutics could be improved by using them in an efficient combination.[302] It was shown that combination of cetixumab with cisplatin provides response rates higher than 50% in squamous-cell carcinoma of the head and neck [303], cetixumab-CTX dual therapy demonstrates 22% of response rates in squamous-cell carcinoma of the head and neck [304], or cetixumab-doxorubicin delivery results 5% response rates in prostate cancer [305].

Similarly, combination of trastuzumab and cisplatin provides receptor enhanced chemosensitivity in metastatic breast cancer with the higher response rate than either single agent alone.[306] Although combination therapy provides much stronger augmentation on the targeted tissue than single agents, it also causes multiple dosing in patients. In order to prevent multiple dosing in patients, combined therapeutics can be attached on a single carrier moiety. With this approach, a compact drug formulation would be obtained through smart carrier systems including image guided delivery or additional targeting applications such as pH, magnetic field. Liao *et al* developed immuno-micelles consisting of cetuximab attached poly(ethylene glycol) block poly(caprolactone) (PEG-PCL). nano-micelles loaded with doxorubicin and superparamagnetic iron oxide to generate drug delivery and magnetic resonance (MR) imaging agent.[307] They used low amount of cetuximab only to target EGFR in A431 cells and to increase the cytotoxic effect of doxorubicin with an efficient targeting. Yan *et al* reported a multifunctional polymeric magneto-nanohybrids for targeted and synergetic therapy of breast cancer.[308] They attached trastuzumab covalently to amphiphilic block copolymer based nano-emulsions which is loaded with doxorubicin and hydrophobic magnetic nanoparticles. A remarkable decrease in tumor volume in xenograft models of NIH3T6.7 cells were observed with these immune nanohybrids compared to doxorubicin-trastuzumab cocktail. There are also several immunotherapeutics studies which target both HER2 and EGFR simultaneously.[309-311] Kwon *et al* generated ^{64}Cu labeled bispecific radioimmunoconjugates consisting trastuzumab Fab and EGF (6.2 kDa, 53 amino acid residues) throughout a PEG₂₄ spacer. [309] Binding strength of radioimmunoconjugate was assessed on MDA MB 231 (EGFR^{mod}, HER^{low}), MDA MB 468 (EGFR^{high}, HER^{neg}), MDA MB 231-H2N (EGFR^{mod}, HER^{mod}), SKOV3 (EGFR^{low}, HER^{high}). They observed a significantly greater uptake of radioimmunoconjugates containing both trastuzumab Fab and EGF compared to their single antibody analogues in MDA MB 231-H2N (EGFR^{mod}, HER^{mod})

cell lines. This study significantly demonstrates that the bispecific receptor targeting is much more efficient than monospecific receptor targeting strategies.

4.2. Experimental

4.2.1. Materials

$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ and $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ were purchased from Merck (U.S.A). Ammonium hydroxide (26%), poly(acrylic acid sodium salt), Bradford reagent, bovine serum albumin (BSA) standard and Potassium Phosphate dibasic anhydrous were purchased from Aldrich (U.S.A.). Herceptin (Roche) And Cetuximab (Medimetriks Pharmaceuticals Inc) were purchased from the pharmacy . Phosphate buffer saline (PBS) and 2-(N-morpholino)ethanesulfonic acid) MES monohydrate buffers were purchased from Biomatik (Canada). Ethylenediaminetetraaceticacid (EDTA) disodium salt was purchased from Multicell (U.S.A.). Potassium Phosphate monobasic was purchased from Riedel-de Haën (U.S.A.) Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysulfosuccinimide (sulfo NHS), DyLight 650 NHS ester, Dylight 488 NHS ester, Traut's Reagent and Slide-A-Lyzer 10k, 20k dialysis cassettes were purchased from Thermo Scientific (U.S.A.) antiHer2 was purchased from eBioscience (U.S.A.) Maleimide and amine heterobifunctionalized polyethylene glycol (Mal-PEG-NH₂) (2k) was purchased from Nanocs (U.S.A.) Vivaspin 20 MWCO 30,000 polyethylenesulfonate (PES) filters were purchased from Sartorius (Germany). Float-A-Lyzer MWCO 300,000 was purchased from Spectrumlabs (U.S.A). N-N dimethylformamide (DMF) was purchased from Merck-Milipore (U.S.A.)

4.2.2. Synthesis of polyacrylic acid coated SPIONs (SPION/PAA)

SPION/PAA was synthesized in water under argon, at 85°C for 1 h with the following molar ratio of the reactants: [PAA]: [Fe^{2+}]: [Fe^{3+}]: [OH^-]-3:2:1:6. After the reaction, mixture was placed on 0.3 Tesla magnetic field for overnight, then the precipitates were removed, and

colloidal suspension was purified with ultracentrifugation using 10 kDa MWCO PES filter. Some amount of product was lyophilized for solid analysis.

4.2.3. Fluorescent tagging of Herceptin and Cetuximab (Her2-dye, EGF-dye)

Commercially available NHS active dyes Cy5 (Dylight 650[®]) and Cy2 (Dylight 488[®]) were separately dissolved in DMF (10mg/mL). Herceptin and Cetuximab were purified from 10 kDa MWCO ultracentrifuge device to desalt the commercial products. 0.5 mg of pure antibodies (antiHer2 (148,000 Da) and antiEGFR (148,000 Da)) were dissolved in sodium borate buffer at pH: 8.55. Antibody and dye solutions were mixed in the molar ratio of [antiHer2] : [Dye] = 1:10 at room temperature for 1h. Overall products were purified with dialysis using 20 kDa MWCO dialysis cassette (Slide-A-Lyzer) against phosphate/EDTA buffer (pH:8.5) at +4°C during 12 h with 4 times buffer refreshment. Protein concentration and molar ratio of protein and dye was calculated as follows.

$$\text{antiHer2 Concentration (M)} = \frac{[A_{280} - (A_{655} \times 0.037)] \times \text{dilution parameter}}{210,000 \times 0.1 \text{ cm}}$$

$$\text{Dylight 655 per mol antiHer2 (mol)} = \frac{A_{655} \times \text{dilution parameter}}{73,000 \times \text{protein concentration} \times 0.1 \text{ cm}}$$

4.2.4. Thiolation of antibodies (Her2-Cy5, EGF-Cy2)

Her2-dye and EGF-dye were mixed with Traut's Reagent at pH 8 at the molar ratio of [1:100] at room temperature for 2h, then the product was purified with dialysis from 10k MWCO dialysis cassette at +4°C for 4h with 6 times buffer refreshment in PBS/EDTA.

Sulfhydryl groups on the antibodies were quantified using Ellman's reagent. 25 µL Ellman's reagent (1mg/mL) was mixed with 25 µL of antiHer2-dye-SH at room temperature for 15

min, then absorbance value at 412 nm was measured to calculate the concentration of thiol moieties with Beer Lambert equation given in the following equation :

$$A = \epsilon \times b \times c$$
$$0.003 = 14,150 \text{ M}^{-1}\text{cm}^{-1} \times 0.1 \text{ cm} \times c$$

4.2.5. Activation of SPION/PAA with maleimide groups (SPION/PAA/MI)

50 mg of SPION/PAA was activated with EDC/sulfo-NHS using the method given for SPION/PAA/AGO2 synthesis, then 3 mg of NH₂-PEG-Mal (2000 Da) was added and mixed for 48h at +4°C and quenched with excess hydroxylamine. Product was purified by washing through 10kDa MWCO amicon ultracentrifuge tube at +4°C for 30 min.

4.2.6. Synthesis of antibody conjugated SPIONs (SPION/Her2-dye, SPION/EGF-dye)

Freshly thiolated Her2-dye and EGF-dye were mixed with maleimide functionalized SPION/AGO2 in PBS/EDTA at pH 7.2 for 1h at room temperature and overnight at +4°C then overall product was purified with the dialysis from 300 kDa MWCO dialysis membrane at +4°C with 4 times buffer refreshment in PBS.

4.2.7. Determination of Herceptin and Cetuximab concentration on SPIONs by

Bradford Assay

Bovine serum albumin protein at 0, 1, 2, 4, 6, 8, 10, µg/mL concentration in PBS was as a standard (Adj. R² = 0.9660). Dialysis product (dialysate) of SPION/AGO2 was concentrated and measured as the analyte. 1 ml of each reference and analyte were mixed with 1 mL of Bradford Reagent for 10 min at room temperature then absorbance at 595 nm was recorded using UV-Vis spectroscopy.

4.3. Characterizations

Photoluminescence spectroscopy (PL) measurements were performed with Horiba Jobin Yvon FluoroMax-3 at room temperature. Absorbance data were collected with UV-3600 Shimadzu UV-VIS-NIR spectrophotometer at room temperature. Dynamic light scattering (DLS) experiments were conducted with Zetasizer Nano Series ZS at room temperature with 173° backscattered angle. Functional groups were investigated using the dried samples and Thermo Scientific Nicolet iS10 ATR-IR. Transmission electron microscopy (TEM) images were taken by Techani G2 F30 at brightfield high resolution (HR) TEM (acceleration voltage 200 kV) using samples deposited on carbon coated Cu-grid from dilute solutions. Vibrating Sample Magnetometry (VSM) measurements were conducted with Cryogenic limited PPMS at ambient conditions. Thermogravimetric analysis (TGA) was made by using TA Q500 under nitrogen atmosphere. X-ray diffraction (XRD) patterns were recorded with Bruker D8 Advance X-ray Diffractometer using Cu-K α radiation.

4.4. Results and Discussion

In this research, the major goal is to improve the receptor targeting potential of SPIONs from the aspect of dual mode affinity to breast cancer. As it is denoted, breast cancer is a heterogeneous disease and it does not have a single type of dysfunction. Although numerous reports identify the breast cancer with Her2 overexpression, this is not the realistic scenario. The type of the receptor overexpression is depends on the subtype of the cancer. Therefore, targeting a singlereceptor overexpression is a good but insufficient strategy, especially for the breast cancer. In order to improve the receptor targeting strategy, we developed SPION-antibody immuno-conjugates for bispecific targeting strategy. In this context, we selected antiHer2 and antiEGFR as immune-therapeutics to target Her2 and EGFR overexpression on

the surface of the several distinct breast cancer cells. Herceptin and Cetuximab were used as their *purified* commercial analogs of antiHer2 and antiEGFR .

During the synthesis of each targeted SPIONs, Herceptin was tagged with far red fluorescent dye while Cetuximab were conjugated with green fluorescent dye. These fluorescent attachments enable us to monitor internalization of each SPION-antibody bio-conjugates and give a clue about the degree of accumulation of each bio-conjugate on the breast cancer cells which differ in the overexpressed receptors on their surface. (Figure 4.1)

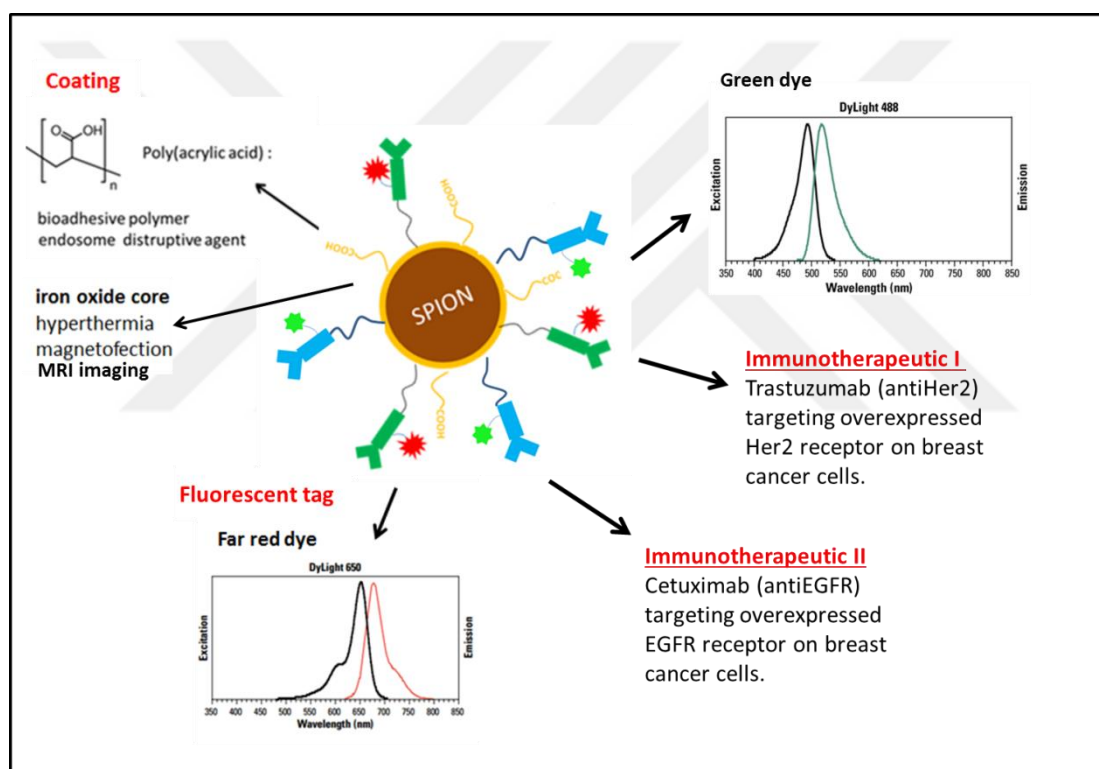


Figure 4. 1 The representative design of the SPIONs with the bimodal emission and targeting capability.

Here, the major goal is targeting rather than therapy. Therefore, antibodies were conjugated at much lower amounts than their therapeutic doses.

SPION immuno-conjugates were designed by using the techniques which were discussed in the previous chapter. Three different compositions were produced in order to conduct a comparative study: SPION for targeting only antiHer2, only antiEGFR, SPIONs targeting both antiHer2 and antiEGFR. (Figure 4.2) Herceptin and Cetuximab were conjugated through a 40 Å PEGylated spacer.

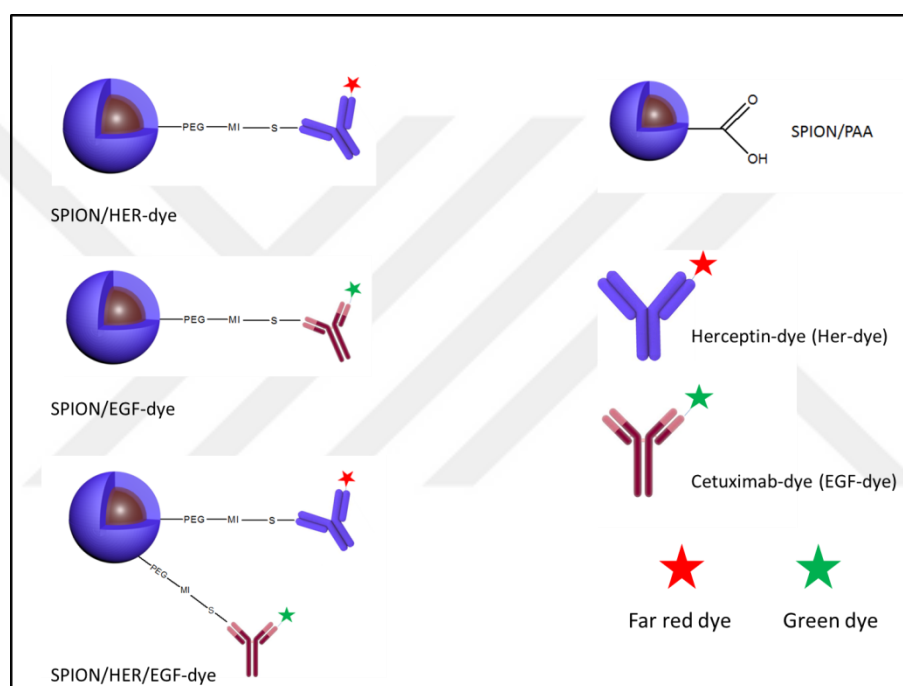


Figure 4. 2 Bispecific SPION immune-conjugates which are decorated with only Herceptin-dye (SPION-Her-dye), only Cetuximab-dye (SPION/EGF-dye) and both Herceptin-dye and Cetuximab-dye (SPION/HER/EGF-dye)

The targeting capability of the constructed SPIONs are tested *in vitro* via cytotoxicity assessment, fluorescence microscopy and fluorescence-activated cell sorting (FACS) experiments. As target cells breast cancer cell lines expressing Her2 and EGFR at different levels and a fibroblast as a negative control were selected (Table 4.2).

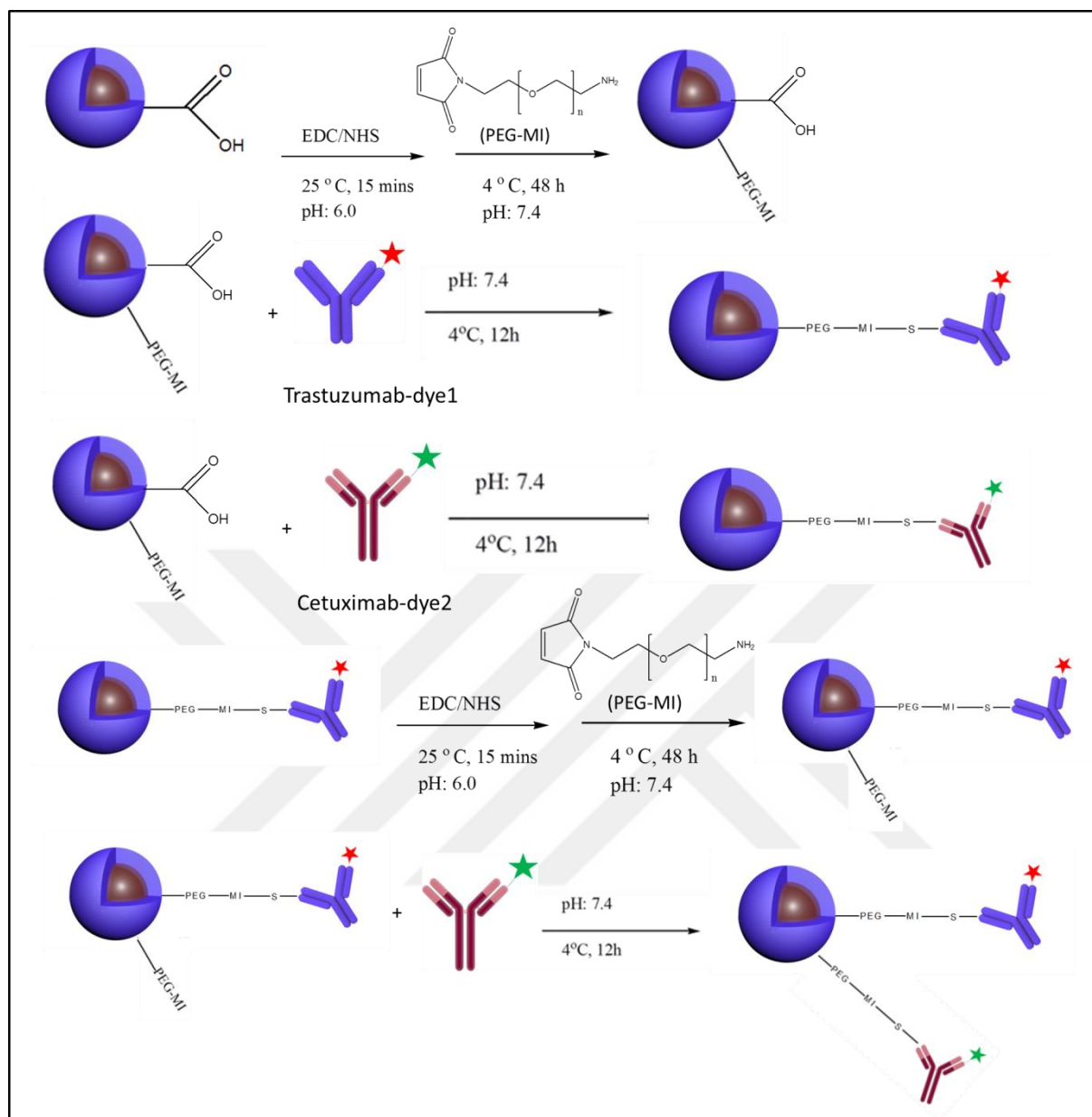
Table 4. 2 Receptor overexpression of the selected cell types

Overexpressed Receptors	MDA-MB-468	MDA-MB-468	SKBR3	Fibroblast
HER2	-	+	+	-
EGFR	+	-	+	-

4.4.1. Synthesis and Characterization of SPION immuno-conjugates

SPION immune-conjugates were synthesized by using the knowledge about the bio-conjugate methods from the previous chapter. Herceptin and Cetuximab were modified fluorescently using the NHS active far red dye Dylight 650 and green dye Dylight 488, respectively. Reaction takes places between the NHS ester of dye units and the amine groups of antibodies. The detailed procedures are given in the Chapter 3.

Fluorescently modified antibodies were conjugated to SPION/PAA particles through the thiol-maleimid chemistry as discussed in detail in Chapter 3. (Scheme 4.1)



Scheme 4. 1 Synthesis of the SPION immune-conjugates which are decorated with only Herceptin-dye (SPION-Her-dye), only Cetuximab-dye (SPION/EGF-dye) and both Herceptin-dye and Cetuximab-dye (SPION/HER/EGF-dye).

Amounts of Herceptin and Cetuximab attached to SPIONs in all three particles were determined by Bradford Assay. The working principle of the assay was discussed in Chapter 3. (Scheme 3.4) The calibration curve created with the reference protein BSA is given in Figure 4.3.

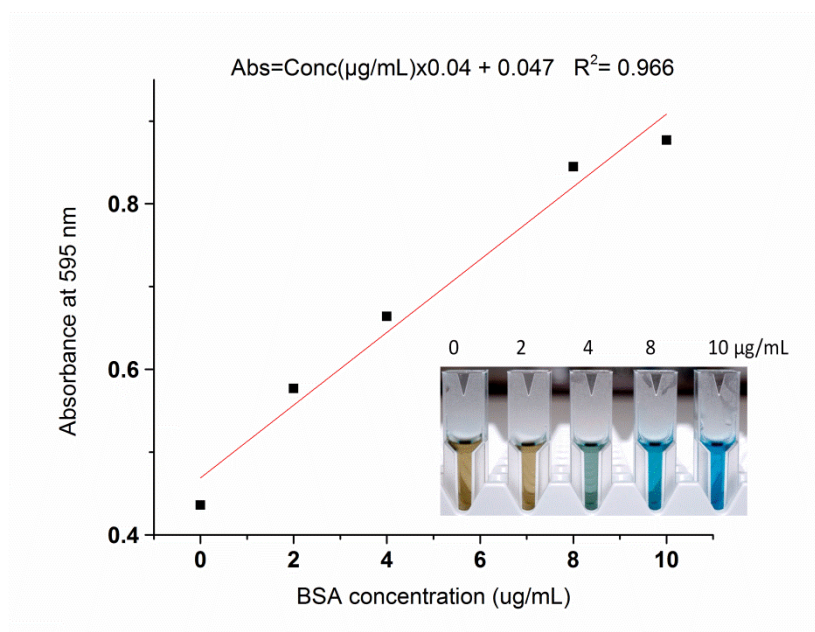


Figure 4. 3 Bradford assay to determine the amount of each Herceptin and Cetuximab on SPION immune-conjugates. A standard curve of BSA concentration ($\mu g/mL$) versus absorbance at 595 nm. (inset : picture showing the interaction of Bradford reagent with BSA at different concentrations)

Table 4. 3 Amount of Herceptin and Cetuximab per mg of SPION immuno-conjugates.

Sample	Herceptin Amount per mg of NP* (μg)	Cetuximab Amount per mg of NP* (μg)
SPION/Her2-dye	7	-
SPION/EGF-dye	-	19
SPION/Her2-EGF-dye	7	21

*Nanoparticle

Table 4.3 shows the amounts of conjugated Herceptin and Cetuximab per mg of each final SPION immuno-conjugates. Although the reactant amounts and the reaction conditions are completely the same, the conjugation efficiency of Cetuximab is 3 times higher than Herceptin. The exact reason behind the difference is not known. Since both Herceptin and Cetuximab are IgG, their three dimensional structure does not differ between each other dramatically. However, amine containing lysine residues on Cetuximab may be spatially more available for the reactions. This condition would facilitate the modifications of Cetuximab and

increase its conjugation efficiency. Although the amount of Herceptin and Cetuximab differs, their amounts do not vary between particles which is critical for a dependable comparison of their targeting efficiency studied in different cell types. 7 μg of Herceptin and around 20 μg of Cetuximab were attached per mg of nanoparticles.

Fluorescent modification of the nanoparticles was done by the similar approach given in the Chapter 3.4.4. (Scheme 3.6) NHS ester active commercial far red and green fluorescent dyes Dylight 650[®] (Cy5) and Dylight 488[®] (Cy2), were attached through the amine units of Herceptin and Cetuximab, respectively. Amount of dye conjugated to antibodies was calculated by using modified Beer Lambert equation as discussed in Chapter 3.4.4. (Equation 10-11) 0.70 mole of Cy2 is attached per mole of Cetuximab, while each mole of Herceptin contains 0.55 mole of Cy5. (Table 4.4) These amounts are enough to get an adequate signal from the antibody without hampering their affinity towards the related receptors.

Table 4. 4 Calculated molar ratio between dye molecules (Cy2 and Cy5) and the antibodies (Herceptin and Cetuximab)

Sample	Abs _{max} *	ϵ_{dye} ($\text{M}^{-1} \text{cm}^{-1}$)	b (cm)	Dilution Factor	Mole of dye per mole of antibody
Cetuximab-Cy2	0.16(@ 280 nm) 0.03(@ 495 nm)	70,000	0.1	10	0.70
Herceptin-Cy5	0.04(@ 280 nm) 0.024(@ 655 nm)	250,000	0.1	10	0.55

*Abs_{max} @ 280,495 and 655 nm belongs to protein, Cy2 and Cy5 respectively.

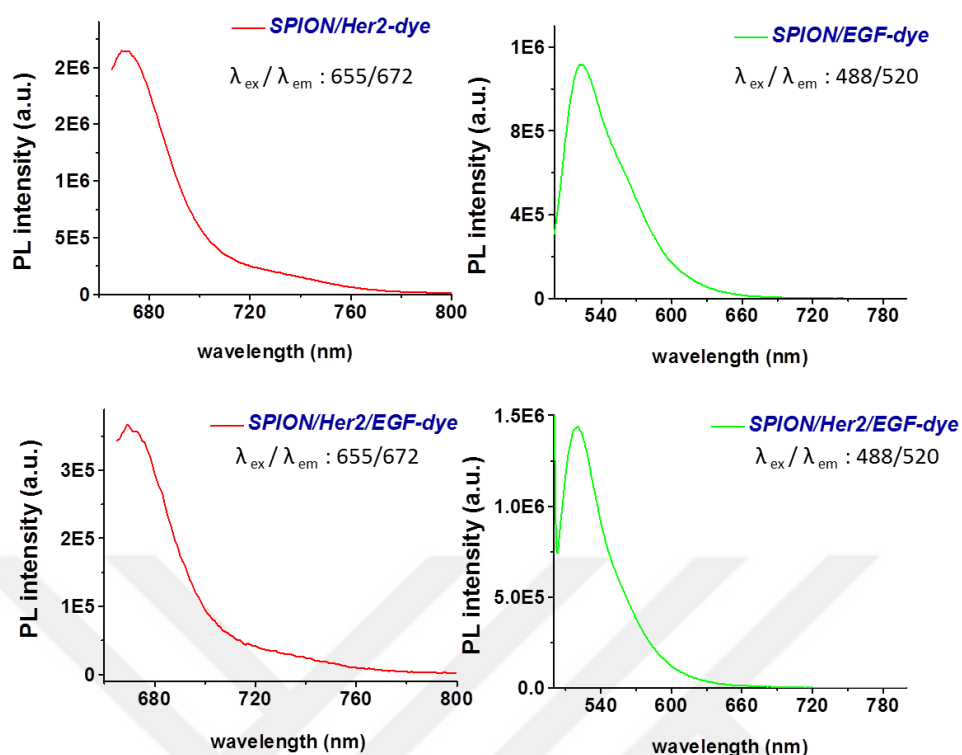


Figure 4. 4 Fluorescence spectra of SPION/Her2-dye ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 655/672$), SPION/EGF-dye ($\lambda_{\text{ex}}/\lambda_{\text{em}} = 488/520$) and SPION/Her2/EGF-dye ($\lambda_{\text{ex1}}/\lambda_{\text{em1}} = 655/672$, $\lambda_{\text{ex2}}/\lambda_{\text{em2}} = 488/520$).

Fluorescence spectroscopy analysis was performed to check the fluorescence signal intensity from each SPION-antibody complex. Sufficient fluorescence intensities were detected at 655 and 520 nm from SPION/Her2-dye and SPION/EGF-dye, respectively. Hence, hybrid SPION/Her2-EGF-dye immuno-conjugates show bimodal emission both at 655 and 520 nm. (Figure 4.4) This dual fluorescence emission capability of the nanoparticles confirms the existence of both Herceptin and Cetuximab on the same construct and namely their bispecific targeting capability.

4.4.2. Hydrodynamic Properties of SPION immuno-conjugates

Hydrodynamic properties of bio-conjugates have vital importance from the aspect of the toxicology and pharmacokinetics.

As it is seen from the Table 4.5, colloidal properties have changed remarkably after the introduction of the hydrophobic maleimid groups to SPION/PAA (SPION/PAA/MI). Hydrophobicity of the maleimid causes aggregation which increases the hydrodynamic size and size distribution (PDI) increases and reduces the zeta potential. Conjugation of the antibodies, reduces the degree of aggregation again except bispecific SPIONs. Charge of the particles stayed as negative. Hydrophobic dyes on the antibodies may affect the aggregation to a certain extent. Although the number based averages of all particles are comparable the bispecific one has the largest PDI with more aggregated particles as represented with the largest PDI (0.51)

Table 4. 5. Hydrodynamic properties of SPION immuno-conjugates.

Sample	Num. average HDR^a (nm)	Int. average HDR^b (nm)	PDI	Zeta Potential^c (mV)
SPION/PAA	19	61	0.30	-22
SPION/PAA/MI	18	98	0.51	-19
SPION/Her2-dye	9	65	0.30	-20
SPION/EGF-dye	21	51	0.21	-12
SPION/Her2-EGF-dye	27	200	0.56	-23

^a Number average size distribution

^b Intensity average size distribution

^c Hydrodynamic size and zeta potential measurements were taken in PBS buffer (pH:7.4)

This polydispersity in the hydrodynamic size of bispecific SPIONs may also arise from the difference in the hydrodynamic size of doubly, single and non-conjugated SPIONs by the antibodies which are statistically all possible in the product. Additionally, colloidal stability and hydrodynamic size range of the nanoparticles are suitable for biological use.

4.5. Conclusions

Targeting strategy is a fundamental approach of the new generation therapeutics. Especially for the diseases with multiple cues, multi-specific targeting approaches have to be developed. Breast cancer is one of the most significant diseases which shows a heterogeneous receptor overexpression. Therefore, we are intended to develop bispecific receptor targeting approach to diagnose and treat the breast cancer more effectively. In this context, SPION immuno-conjugates were decorated with both Herceptin and Cetuximab antibodies to target the HER2 and EGFR overexpressed receptors which regulate cell signaling, growth and proliferation. Cy5-tagged Herceptin and Cy2 tagged Cetuximab were linked to SPIONs using thiol-maleimide chemistry. SPIONs conjugated with both antibodies should be capable of targeting both antiHer2 and antiEGFR. Beside their specific molecular targeting, they can be directed under magnetic field which provides magnetic targeting, as well. SPION/Her2-dye and SPION/EGF-dye and dually tagged SPION/Her2-EGF-dye contain sufficient degree of antibody to target Her2 and EGFR receptors of breast cancer cells. Fluorescent dyes allows optical tracking of these nanoparticles. . Bispecific SPION immuno-conjugates are anionic, colloidally stable (-23 meV) and small enough to circulate in the body (200 nm, intensity average distribution, 27 nm number average distribution).

The ultimate goal of bispecific SPION immuno-conjugates is to strengthen the breast cancer targeting capacity of the superparamagnetic nano-carriers. In order to confirm that, SPIONs targeting only one type of receptor have been also produced. (SPION/Her2-dye and SPION/EGF-dye) to be tested on several breast cancer cells and fibroblast which differ in the level of overexpression of Her2 and EGFR. In vitro studies of SPION immuno-conjugates including FACS and MTT analysis are ongoing in collaboration with the Molecular Biology Department, Haliç University. If continuing *in vitro* studies would support the efficient and bispecific targeting capability of SPIONs, it will increase the efficiency of early diagnosis of

the breast cancer and improve the therapeutic outcome by overcoming the tumor heterogeneity which is a major obstacle in targeted theranostics.



CHAPTER 5

5. CONCLUSIONS

SPIONs are highly promising biomedical materials in clinical applications such as drug delivery, gene delivery, MRI, hyperthermia and tissue engineering due to their high biocompatibility, magnetic responsivity, ease of preparation and suitability for further chemical modifications. However, SPION based compositions still need significant improvements in biocompatibility and targeting efficiency. Three major improvement needs are attached in this thesis. (1) Diagnostic potential of SPIONs via a second mode of detection such as optical detection, beside the MRI, which is highly desired both in vitro and in vivo. (2) Novel and highly biocompatible SPION based miRNA delivery agents. (3) Targeted SPIONs which can overcome the tumor heterogeneity.

In Chapter 2, a novel self-luminescent-superparamagnetic gene delivery system was reported. It was shown that the poor blue luminescence of PEI can be improved 1200 times if it is adsorbed on the SPION via aqueous precipitation of iron salts which causes oxidation of part of the amine groups in PEI. . Origin of the luminescence of polyamines was investigated by using various spectroscopic techniques. Combination of amine oxidation, surface adsorption of bPEI restricting the mobility coupled with final acidification are suggested as the major affecters of such strong emission of bPEI-SPION. Suggested factors in the literature such as relative proximity of amines and exciton formation may contribute as well, which can be understood better when all similar systems are studied together, in the future studies. Besides, these self-luminescent iron oxide nanoparticles are superparamagnetic and they can also produce a remarkable T₂ signal at the concentration which generates a strong blue luminescence. This indicates the

preservation of superparamagnetic nature of the iron oxide core and potential for both optical and magnetic detection. Indeed, strong intracellular optical signal generated by bPEI-SPION supported the utility of such luminescence at least in the in vitro studies. In addition, in a simple, proof-of-concept experiment, it was shown that bPEI-SPION can bind (37 weight %) a model oligonucleotide, PIC, and effectively deliver to HeLa cervical cancer cells which was indicated by significant cell death at the concentrations where bPEI-SPION itself was not toxic. Hence, chemical changes occurred on bPEI during the synthesis did not hamper its traditional transfection potential. Overall, these findings are crucial in evaluation of luminescence obtained from similar systems especially which contains sort of regular distribution of amines, and especially important for those who evaluate such systems for bio-imaging/gene delivery purposes. In Chapter 3, design and development of multifunctional SPION-protein complexes are proposed as biocompatible, non-cationic miRNA delivery vehicles. Small and stable PAA coated SPIONs are decorated with natural miRNA carrier protein, AGO2, to bind and deliver therapeutic miRNA to breast cancer via attached antiHer2. Since the current gene delivery technology suffers from the toxicity of cationic nature of gene delivery agents, association of AGO2 protein into the SPION complex as miRNA carrier creates a non-toxic gene carrier model, which is a completely new gene-delivery platform.

SPION bio-conjugates provide a far red fluorescent imaging capability due to dye-conjugation on the Her2 targeting ligand, antiHer2 which allows particle tracking in vitro and in vivo. Iron oxide core of this system will be also utilized for magnetic targeting and also for hyperthermia as an adjuvant therapy, in the following studies being conducted at Sabanci University by Prof. Ali Kosar

AGO2 and fluorescently modified antiHer2 were carefully conjugated SPIONs to create versatile SPION bio-conjugates using the bio-conjugation chemistry. The major goal of the developed bio-conjugation strategies is to produce the designed SPION based miRNA carrier complex using the most feasible procedure which preserves the intrinsic characteristics of each building block. For this purpose, some key arrangements were taken into account to optimize the bio-conjugate design. In order to obtain an optimum spatial arrangement, AGO2 was attached in a protected manner to keep the therapeutic miRNAs from the enzymatic degradation. antiHer2 was attached with a flexible and biocompatible PEG linker to enhance its interaction with Her2 receptor. Dye was attached to antiHer2 so that its luminescence would not be quenched with SPION. Also a far red dye was selected to minimize the quenching by SPION, as well. Synthesized nanoparticles are proved to be small enough (49 nm) to escape from the RES, colloidally stable in the pH range 4-8 (-24 meV) which can potentially circulate in the blood vessel without any coagulation. Hence, SPION/AGO2/antiHer2-dye bio-conjugates are brightly red fluorescent which can be tracked under fluorescence monitoring. Around 124 AGO2 and 24 antiher2 proteins were calculated per SPION using Bradford assay and the hydrodynamic properties of the nanoparticles. *In vitro* MTT assay and *in vivo* blood biochemistry analysis performed by Prof. Devrim Gozuacik show that nanoparticles are almost non-toxic to the concerned breast cancer cells SKBR3, MDA-MB-453 and nude mice models at the dose of 0.15 mg and 48 h time exposure. Highly selectivity uptake of SPION-protein complexes into the Her2 overexpressed breast cancer cells SKBR, MDA-MB-453 and their tumor models on nude mice were demonstrated, as well. SPION-protein complex achieves a successful *in vitro* transfection of the miR376b mimics especially to the MDA-MB-453 cells (around 20%). Overall, newly developed ultrasmall, colloidally stable and multifunctional SPION-protein bi-conjugates have a

great potential as nanotheranostic for a successful and non-toxic miRNA delivery. The *in vivo* demonstration of its therapeutic effect is an ongoing part of the project.

In chapter 4, SPIONs decorated with two different affinity antibodies, Herceptin and Cetuximab, were produced to overcome the failures in targeted detection and therapy of breast cancer due to the heterogeneity of the tumors. Bispecific affinity approaches provide an enhanced targeting strategy especially for the diseases with multiple molecular symptoms. Breast cancer is one of these types of diseases with the heterogeneous receptor overexpression and needs advanced molecular targeting strategies. Therefore, a bispecific receptor targeting approach was developed to treat the breast cancer more efficiently. For this purpose, we developed SPION immuno-conjugates with the association of Herceptin and Cetuximab antibodies to target the overexpressed receptors HER2 and EGFR. Fluorescent modifications of Herceptin and Cetuximab were done by the linkage of Cy5 and Cy2 dyes, respectively. Mono- and dual tagged SPIONs were prepared for a comprehensive study with sufficient degree of antibodies to target Her2 and EGFR receptors of breast cancer cells per particle (7 μ g of Herceptin per mono and dual tagged SPIONs, 19 μ g and 21 μ g of Cetuximab per mono and dual tagged SPIONs respectively)

Additionally, SPION immuno-conjugates allow the fluorescence imaging due to the Cy5 and Cy2 attachments. Bispecific SPION immuno-conjugates are anionic, colloiddally stable (-23 meV) and small enough to circulate in the body (200 nm, intensity average distribution, 27 nm number average distribution).

In order to show the enhanced targeting potential SPION immuno-conjugates, internalization levels of the nanoparticles were analyzed using several breast cancer cells and fibroblast which differ in the level of overexpression of Her2 and EGFR. Theoretically, we expect that

doubly conjugated SPIONs (SPION/Her2/EGF-dye) would show the most efficient uptake by the SKBR3 cells overexpressing both Her2 and EGFR

receptor. In vitro studies of SPION immuno-conjugates are being conducted at Haliç University by Dr. Ayyub Ebrahimi. Generated SPION immuno-conjugates are potentially stronger candidates among their counterparts in the targeting strategies for especially breast cancer.

Overall, a conceptual and methodological route is followed to discuss the improvements in the theranostic potential of SPIONs within this thesis. We believe that the studies in the scope of this thesis significantly contribute to the immature sides of the SPION based cancer theranostics by offering reasonable solutions to alter the massy designs, high toxicities and poor molecular targeting strategies.

5.1. Publications and Conferences

Ö. Ünal, H. Yağcı Acar, M. Koçak, Y. Akkoç, D. Gözüaçık, Multifunctional Protein Coated Nanoparticles for Targeting Intracellular miRNA in Breast Cancer Cells, 14th International Conference on Nanosciences & Nanotechnologies (NN17) Thessaloniki, 4-7 July 2017. (oral presentation)

Özlem Ünal, Havva Acar Yağcı, Rouhollah Khodadust, Emre Erdem, Barış Yağcı, “*Self luminescent iron oxide nanoparticles for imaging and gene delivery*”, 30th European Colloid and Interface Society Conference, (ECIS) Rome, Italy, September 4-9, 2016 (oral presentation)

Unal, Ozlem; Khodadust, Rouhollah; Durmusoglu, Emek; Erdem, Emre; Yagci, Mustafa; Yurtsever, Ersin; Yagci Acar, Havva “Discovery of the Exceptionally Strong Luminescence from Polyethyleneimine When Coated on the Iron Oxide Nanoparticles” (*in preparation*)

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