FABRICATION OF CLEAVABLE COVALENTLY CROSSLINKED COLLOIDOSOMES

by

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Dedicated to my family

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ABSTRACT

In this study, cleavable microcapsules were obtained using self-assembly and crosslinking of magnetic nanoparticles at the oil-water interface. To achieve this, iron oxide nanoparticles coated with oleic acid were synthesized and they were coated with three different linkers including azide and alkyne parts, one of which contained a disulfide bond linkage. Since disulfide bonds can be degraded with thiol compounds, they render these microcapsules cleavable. A water in toluene emulsion system including both nanoparticles and Cu(I) catalyst was prepared. By shaking the emulsion, microcapsules were fabricated via Cu(I) catalyzed Huisgen reaction involving cycloaddition of azides and alkynes. Thus, stable microcapsules made through covalent crosslinking of coated iron oxide nanoparticles were obtained. These microcapsules were characterized using microscope and SEM images. Also, FITC labeled dextran was encapsulated within these microcapsules and their release in the presence of dithiothreitol was demonstrated using fluorescence microscopy.

ÖZET

Bu çalışmada ilk olarak oleik asit kaplı demir oksit nanoparçacıklar sentezlendi. Daha sonra üç farklı bağlayıcı ve oleic asit arasında gerçekleşen yer değiştirme reaksiyonu sonucunda bağlayıcılar ile kaplandılar. Üç bağlayıcıdan bir tanesi diğerlerinden farklı olarak disulfit bağı taşımaktadır. Disülfit bağı tiol içeren maddeler ile bozulabilir. Bu özellik disülfit bağı içeren mikrokapsüllere daha sonra kırılabilirlik özelliği kazandırır. Yer değiştirme reaksiyonları ile demir oksit parçacıklar kaplandıktan sonra toluen içinde demir oksit parçacıklar, su içinde Cu(I) kataliz emülsiyonu kullanılarak azid ve alkin grupları arasında bir klik reaksiyon meydana getirerek içlerinde su bulunan mikrokapsüller elde edilmiştir. Oluşturulan mikrokapsüller pek çok alanda kullanabilir. Bu alanlardan bir tanesi biyomoleküller için taşıyıcı görev görmeleridir. Bu amaçla mikrokapsüllerin içine bir floresans boya konmuş ve oluşturulan mikrokapsüllerin bu özelliğe sahip oldukları boyanın mikrokapsüllerden salınımı kontrol edilerek gösterilmiştir.

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LIST OF ACRONYMS/ABBREVIATIONS

Au NPs	Gold Nanoparticles
$CDCl_3$	Deuterated Chloroform
CTA	Chain Transfer Agent
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
Dex-FITC	Fluorescein Isothiocyanate-Dextran
DMAP	4-Dimethylaminopyridine
DMSO	Dimethyl Sulfoxide
DTT	Dithiothreitol
EtOAc	Ethyl Acetate
EtOH	Ethyl Alcohol
FT-IR	Fourier Transform Infrared
GSH	Glutathione
KI	Potassium İodide
MCs	Microcapsules
Na_2SO_4	Sodium Sulfate
NaAsc	Sodium Ascorbate
NaCl	Sodium Chloride
NaN_3	Sodium Azide
NMR	Nuclear Magnetic Resonance
NPs	Nanoparticles
PGMA	Poly (Glycerol Monomethacrylate)
PHPMA	Poly (2-Hydroxy Propyl Methacrylate)
ROMP	Ring Opening Metathesis Polymerization
SEM	Scanning Electron Microscope
TEM	Transmission Electron Microscope
UV	Ultraviolet

1. INTRODUCTION

1.1. Microcapsules or Colloidosomes

Microcapsules or colloidosomes are permeable spherical capsules composed of colloidal nano- or micro- particles. Colloidal microcapsules are useful materials for a number of area because of their high surface area, large internal volume and stability [1]. Using them as drug delivery agent, catalyst support and encapsulants are only a few of these areas [2]. In addition to these properties, the permeability, mechanical strength and biocompatibility of capsules obtained from nanoparticles can be altered by changing the core or ligands of the colloidal building blocks [3]. As an illustration microcapsules composed of polymeric and metal nanoparticles are shown below (Figure 1.1).

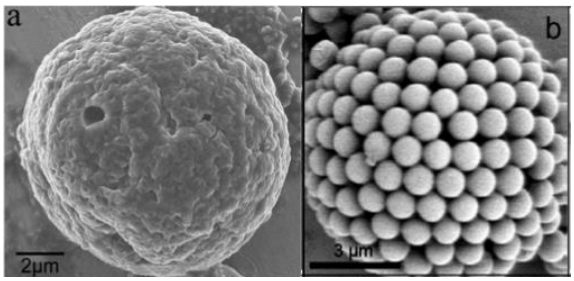

Figure 1.1. Colloidosomes (a) polystyrene melamine formaldehyde containing at the oil water interfaces [4]. (b) containing polystyrene particles at the toluene water interfaces [1].

There are two widely used methods that are used to obtain stable and multifunctional microcapsules. One of them is that coalesced lipids and polymers spontaneously. The other way is to prepare an emulsion and stabilize it with colloidal particles at the water-oil interface [5]. In latter method, metal and polymeric nanoparticles have been used. Nano- and microcapsules made using inorganic nanoparticles are attractive since they benefit from the electrical, magnetic and optical feature of nanoparticles. Because of this reason fabrication of microcapsules using gold or iron oxide nanoparticles have been extensively investigated.

1.1.1. Molecular Self Assembly

In order to fabricate nano- and microcapsules using self-assembly at oil-water interface, it is important to use building blocks that have some intermolecular interactions between them. Such intermolecular interactions will increase the stability of these constructs due to increased cohesive forces between particles. The molecular self-assembly process involves spontaneous organization of molecules. Without an effect or administration from an external source, self-assembly between molecules can occur through various non-covalent interactions. Hydrogen bonding, metal ligand coordination, hydrophobic forces, van der Waals forces, pi-pi interactions and electrostatic effect shown in the table 1.1 are a few of these interactions [6].

Table 1.1 Strength of non-covalent interactions.

Type of Non-Covalent Interactions	**Strength (kJ mol^{-1})**
Hydrogen Bond	2-30
Metal Ligand	0-400
Hydrophobic	<10
Van Der Waals Forces	0.1-1
Pi-Pi Interactions	0-10
Electrostatic Effect	1-20

Microstructures constructed from self-assembly of molecules can be seen in nature. For example, many biological systems like the phospholipid bilayer cell membrane and liposomal constructs are arranged through self-assembly (Figure 1.2).

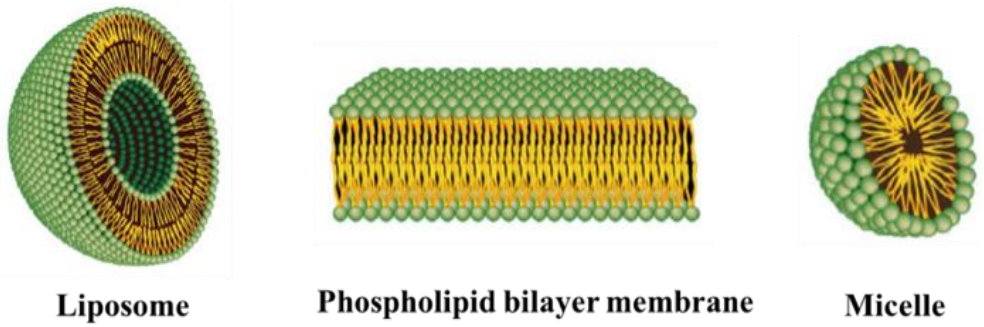

Figure 1.2. Self-assembled structures formed by self-assembly [56].

Scientists have been inspired by nature to employ self-assembly process as a tool to construct micelles, microspheres, vesicles, self-assembled monolayers or colloidal microcapsules [7].

Self-assembled nano- and microparticles at the emulsion interface can be used for making nano and micro scale assemblies, capsules and ultra thin sheets [1]. In order to make microcapsules with numerous functionalities, several approaches based on self-assembly and templates were introduced [8].

1.1.2. Building Blocks for Colloidal Microcapsules

Colloidal particles play an important role as building blocks for outer surface of colloidal microcapsules. Microcapsules constructed using well-defined colloidal particles provide an alternative option based on polymer and lipid systems [9]. One of the methods widely used for fabrication of these MCs involves stabilizing emulsions via self-assembled nano or micro particles at the emulsion interface. There are many kinds of colloidosomes which have generated, such as liposomes [10], polymersomes [11,12] and polyelectrode capsules [13,14].

Nanoparticles (NPs) can be used as receptor to outer surface triggers (e.g. magnetic fields or laser) in order to control the release from fabricated materials [15]. Besides, the assembled colloidal particles at the liquid liquid interface may end up with well-organized structures with unique electronic, magnetic and optical characteristics arising from the assembly [16]. These characteristics of MCs can be controlled by changing their size and macro and nanoscopic level effects [1,17].

1.1.3. Iron Oxide Nanoparticles

Magnetic particles size (nano or micro) are desirable for lots of area such as magnetic recording, biological and medical applications [18]. One of the up and coming particle is different iron oxide nanoparticle types; magnetite (Fe_3O_4), maghemite (γ-Fe_3O_4) and hematite (α-Fe_3O_4). Owing to their small size and super-

paramagnetic manner, these are often used as materials for drug delivery, hyperthermia, magnetic resonance imaging (MRI) contrast agent [19], magnetic cell labeling and sensors [20-25]. Additionally, iron oxide nanoparticle's non-toxicity and high biocompatibility make them favorable for biotechnology fields [21].

Since NPs have large surface area to volume ratio, they have tendency toward agglomerate to reduce their surface energy [22]. Thereby the magnetic nanoparticles need to be coated with a surfactant for the purpose of decreasing their surface energy. This surface coating of nanoparticles also plays a key role on their properties and applications [23]. Oleic acid is the most preferred surfactant to modify the surface of magnetic particles hence it has higher affinity towards the surface of magnetic particle compared to other surfactants [24,25].

In the last decades, several methods have been introduced for the fabrication of iron oxide NPs based on stability, biocompability, control of shape and monodispersity. Co-precipitation, thermal decomposition, hydrothermal synthesis, microemulsion and sonochemical synthesis are some of the most conventional methods to synthesize iron oxide nanoparticles. All types can fabricate high quality nanoparticles but co-precipitation method is the most common way to produce iron oxide NPs owing to its simplicity and scalability. It utilizes mixing of ferric and ferrous ions with 1:2 equivalents at room temperature or at high temperature. The type of salt used such as chlorides, the ferric and ferrous ions ratio, temperature, pH value, ionic strength of the environment and the other reaction features are important because they define that how iron oxide NPs will be in terms on the diameter and shape [26].

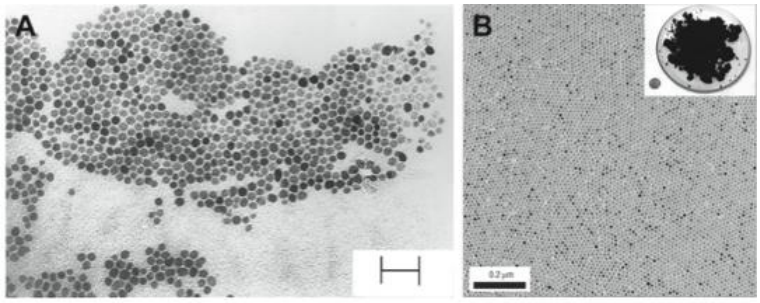

Figure 1.3. (a) Fe_3O_4 NPs were formed via co-precipitation method (scale bar: 30nm). (b) Fe_3O_4 NPs were formed via thermal decomposition method.

Figure 1.3. shows two examples which were synthesized NPs by using different methods and for different applications [27].

1.1.4. Pickering Emulsions

Routes to colloidal microcapsules mostly rely on the self-assembled colloidal particles at the interface to minimize interfacial energy between two immiscible liquids, typically oil and water. The first self-assembled formation was disclosed for the first time by Ramsden in 1903 [28]. However, it was termed as Pickering emulsions after S.U. Pickering published the article in 1907 [29]. After that, this process caught little attention till 1990. Since Velev and coworkers exhibited this approach's potential to use oil-water emulsions as template for obtaining of novel colloid-based materials [30], an increased attention has been paid towards Pickering emulsions.

Yoe Li and co-workers synthesized plasmonic colloidosomes based on Au nanospheres by using a reverse water-1-butanol emulsion system (Figure 1.4) [31]. As can be seen from SEM analysis, colloidosomes based on Au NPs were obtained and most of them showed spherical shapes and only a few were damaged. From some images it can be clearly inferred that these colloidosomes have hollow interiors.

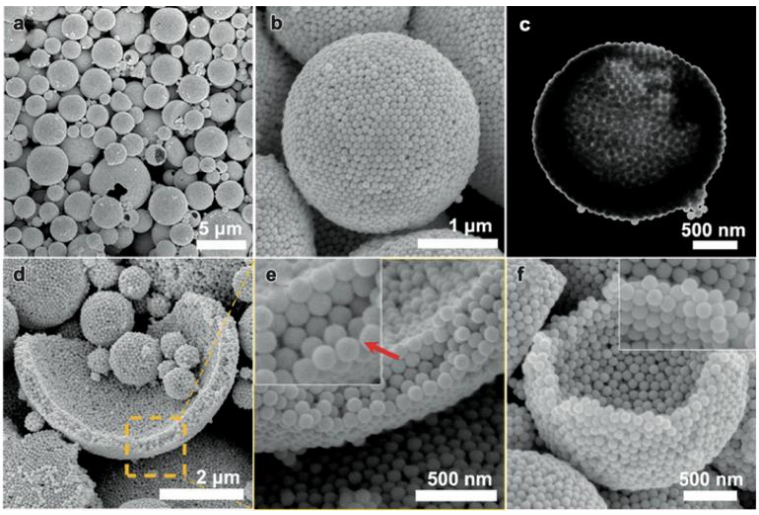

Figure 1.4. SEM images of the self-assembled Au PCs (a, b). Scanning-TEM image of single Au PC (c). SEM images of the broken Au PCs with different size (d, f).

To understand the reason for stabilization of Pickering emulsion, Pieranski demonstraded the drop in interfacial energy is connected to both effective radius (r) and interfacial tensions of particles [32]. The basic equation (1.1) is:

$$\Delta E = -\frac{\pi r^2}{\gamma_{o/w}}\left[\gamma_{o/w} - \left(\gamma_{p/w} - \gamma_{p/o}\right)\right]^2 \quad (1.1)$$

Where $\gamma_{p/o}$ = interfacial energy of particle-oil systems, $\gamma_{p/w}$ = interfacial energy of particle-water systems and $\gamma_{o/w}$ = interfacial energy of oil-water systems.

Stabilization of particles at the oil water interface is strongly controlled by their size. That means that for smaller NPs energy gain will be lower and the assembly will not be as stable when compared to larger NPs [33]. As a result, larger particles can be absorbed at the oil water interface irreversibly and produce stable emulsions while smaller NPs can be absorbed much more weakly.

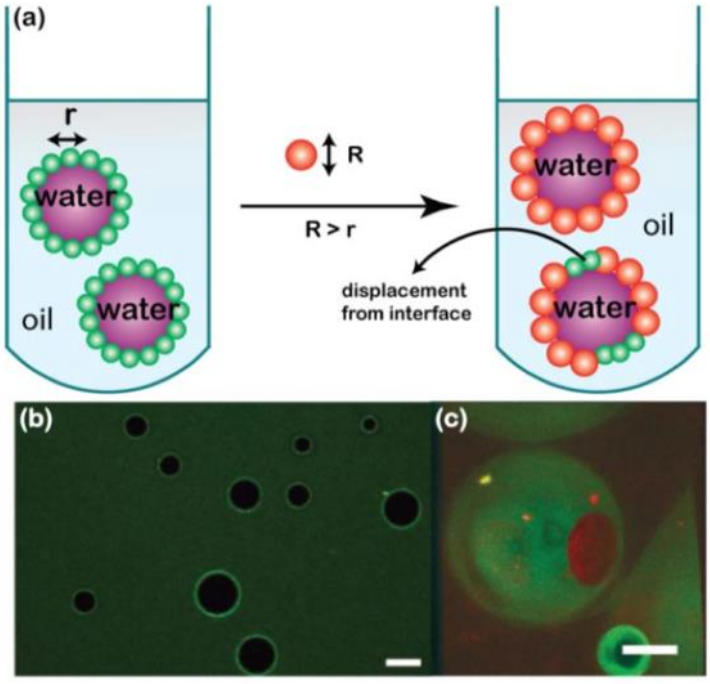

Figure 1.5. Schematic representation of particles migration via size selectivity at the surface of droplets (a). Confocal microscopy images of droplets composed of smaller CdSe particles (b) and after addition of larger particles (c).

Russell and co-workers showed the NPs adsorption at the liquid liquid interface via size selectivity by using fluorescent CdSe quantum dots. At first, in order to make a stable emulsion, they took smaller CdSe NPs which possessed green fluorescence and added them into the oil phase. Then, they added larger CdSe NPs which fluoresced red to the media. After observed that larger NPs adsorbed on the droplets and replaced with the smaller ones (Figure 1.5) [34].

Wettability of NPs is other key factor for the assembly at liquid-liquid interface. Using Young's equation, the relation between interfacial energies and contact angle (1.2) can be obtained;

$$\cos\theta = \frac{\gamma_{po} - \gamma_{pw}}{\gamma_{ow}} \qquad (1.2)$$

According to Young's equation, γ_{po}, γ_{pw} and γ_{ow} are the interfacial tensions of particle-oil, particle-water and oil-water interface, respectively where the θ = contact angle. So, when a particle has contact angle less than 90°, it shows hydrophilic characteristic, in other words, the particle will be wetted mostly by water ($\gamma_{po} > \gamma_{pw}$). On the other hand, if the particle has contact angle larger than 90°, than particle will be wetted by oil more than water ($\gamma_{po} > \gamma_{pw}$). Only intermediate contact angle value can provide the most stable Pickering emulsions, in that situation θ = 90° and $\gamma_{po} = \gamma_{pw}$ (Figure 1.6).

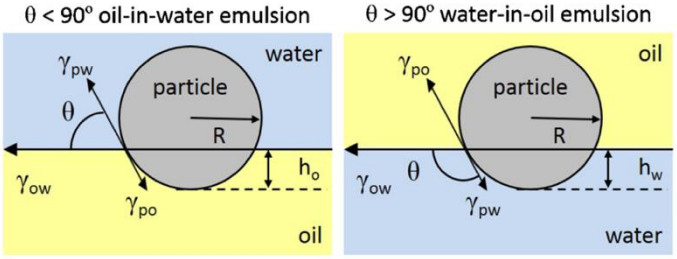

Figure 1.6. Wettability of particle in an oil-water interface [35].

Not only the size and wettability but also interactions between particles at the emulsion interface acts a crucial role in the self-assembly part. Lots of interfacial

forces including capillary, solvation, electrostatic, van der Waals and fluctuation forces are connected with conducting these interactions at the interface [36].

1.1.5. Crosslinking NPs at the Oil Water Interface

Crosslinking is the process of chemically bonding of two or more participants by a covalent bond. In general, these linkages are stable mechanically and thermally i.e. once they form, they are difficult to break. Therefore, often, cross-linked products are not reversible.

The most direct way for crosslinking NPs at the liquid-liquid interface is through their surface ligands via chemical bonds or interactions. There are two types of crosslinking methods; covalent interactions between NPs and non-covalent interactions between NPs.

1.1.5.1. Covalent Interaction between Nanoparticles. Covalent crosslinking between ligands produces distinguishable mechanical coherence and stability for the NP assemblies. In a work belonging to Lin and co-workers, ring opening metathesis polymerization (ROMP) was used to crosslink appropriately modified CdSe-ZnS NPs [37]. Here, after a ligand based on nonbornene was coated on CdSe-ZnS NPs, then they were coalesced at the liquid liquid interface via shaking vigorously and a water soluble Grubbs catalyst (generation II) was introduced to obtain cross-linked capsule formation. Thus, the polymerization worked at room temperature and produced stable, defect-free MCs at the liquid-liquid interface as confirmed using fluorescence confocal microscopy (Figure 1.7).

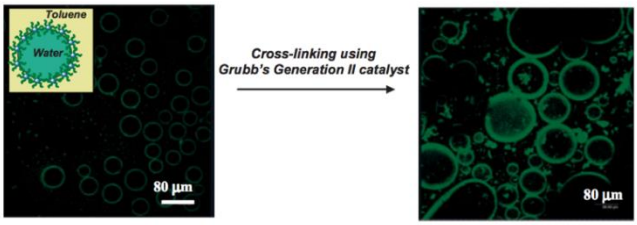

Figure 1.7. Assembly of quantum dots at toluene/water interface before and after ROMP polymerization.

As a alternative study, Rotello and co-workers formed stable colloidosomes composed of FePt NPs at the emulsion interface by using coordination chemistry [38]. In their work, first, they synthesized FePt NPs and then coated the NPs with terpyridine thiol (terpy-SH). By preparing an emulsion which has a water phase containing Fe(II) tetrafluoroborate hexahydrate and a toluene phase containing FePt NPs, self-assemblies of NPs were obtained and they were cross-linked by using transition metal mediated interactions (Figure 1.8). However, formation of colloidosomes were unstable if there was no Fe(II) salt in the environment. At the end, the main advantage of this method is quick formation of cross-linked structure at ambient conditions.

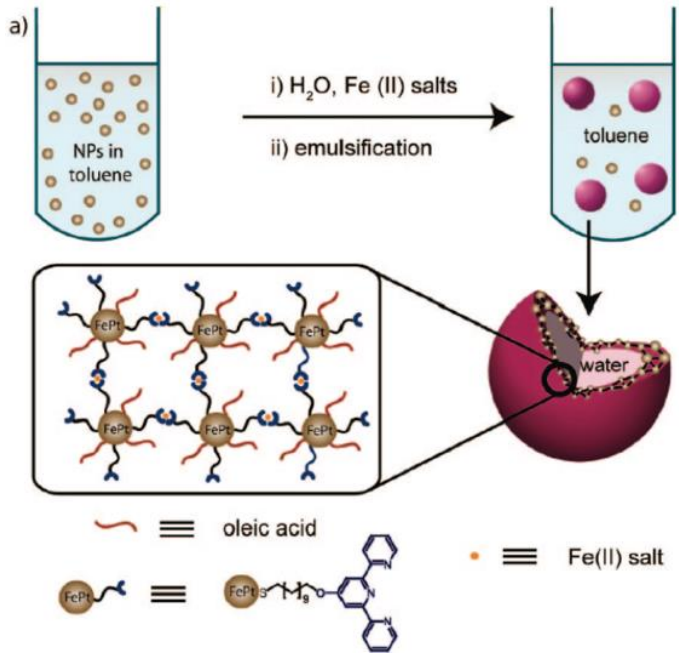

Figure 1.8. Illustration for fabrication of FePt NPs water in toluene interface.

In addition, crosslinking by using a metal, Rotello and co-workers builded up covalent approaches so as to stabilize NPs solution. In order to crosslink NPs at the interface, the highly efficient cupper (I) catalyzed Huisgen-type [3+2] “click” cycloaddition reactions were used under ambient conditions. In this study, Fe_3O_4 NPs were synthesized and they were functionalized with two different linkers via place exchange reaction. One of which linkers had alkyne group and the other had azide group. After that, NPs bearing alkyne and azide were assembled at the liquid liquid

interface with shaking the mixture vigorously (Figure 1.9) and thanks to cooper (I) catalyst which was added during preparation of emulsion, NPs were covalently binded to each other by using click reaction (Figure 1.10).

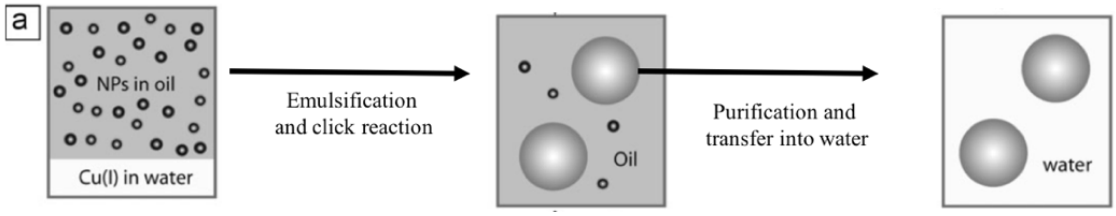

Figure 1.9. Formation of magnetic colloidosomes through self-assembly at the interface and crosslinking of NPs via click chemistry.

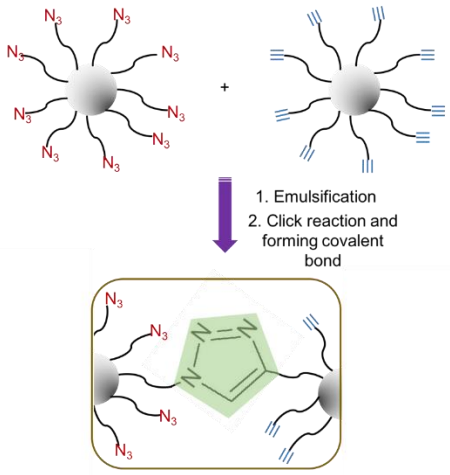

Figure 1.10. Formation of covalent bond between colloidosomes.

This kind of crosslinking has two major advantages. One of them is click reaction between alkyne and azide side on NPs. It is generally selective and is not affected from existance other functional groups and environmental conditions like pH and solvents. The other advantage is that this process makes crosslinking of NPs denser on the external surface of MCs which ends up with highly stable microcapsules and they can be transferred into a homogenous solvent [39].

1.1.5.2. Non-covalent Interaction between Nanoparticles. Non-covalent interaction between NPs and dynamic nature of the self-assembled NPs has a potential to create

novel functionality such as catalysts, sensors and devices. In a recent work, Sanyal and Rotello introduced stable microcapsules fabricated by using “host-guest” interaction at the liquid liquid interface [5]. In this study, water soluble β-cyclodextrin coated gold NPs as a host molecule and organosoluble adamantyl functionalized gold NPs as a guest molecule were used to create microcapsules (Figure 1.11) and they were cross-linked via specific host-guest interactions at the oil-water interface (Figure 1.12).

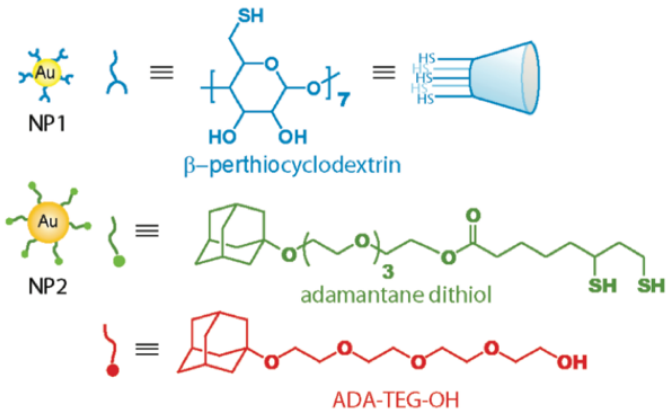

Figure 1.11. Structures of ligands and NPs.

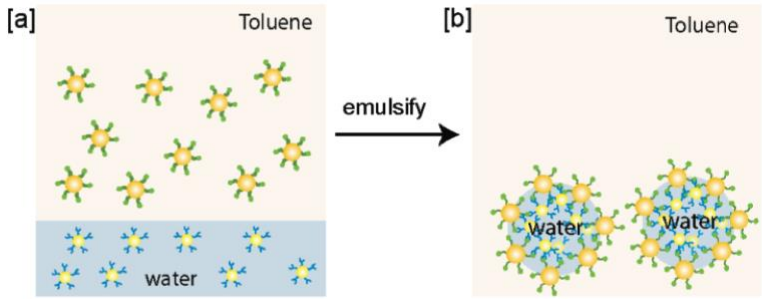

Figure 1.12. (a) NP 1 and NP2 dispersed in water and toluene, respectively. (b) Fabrication of colloidal microcapsules via recognition mediated cross-linking.

1.1.6. Crosslinking NPs at the Dispersed Phase

Colloidal microcapsules composed of NPs have a problem which is their poor stability when they are transferred into a homogenous solvent. One of the solutions is

crosslinking NPs at the oil-water interface. Another alternative solution is solidification or gelation.

In a recent work, Möhwald and co-workers fabricated microcapsules by using agarose solution as the dispersed phase in toluene. With the aqueous solutions of agarose and the toluene dispersion of Fe_3O_4 NPs at 70 ^oC, an emulsion was prepared [40]. It was done at the high temperature because of agarose gelation. When the mixture was cooled down until room temperature, agarose gave the gelation at the aqueous phase which makes microcapsules were more stable at the interface. Then, by using an external magnetic field, stabilized NPs were collected, washed with ethanol and redispersed in water (Figure 1.13). On the other hand, in the absence of agarose gel, after washing with ethanol and redispersing in water, MCs collapsed. This showed that the agarose gel is necessary for maintaining the stability of MCs against environmental changes.

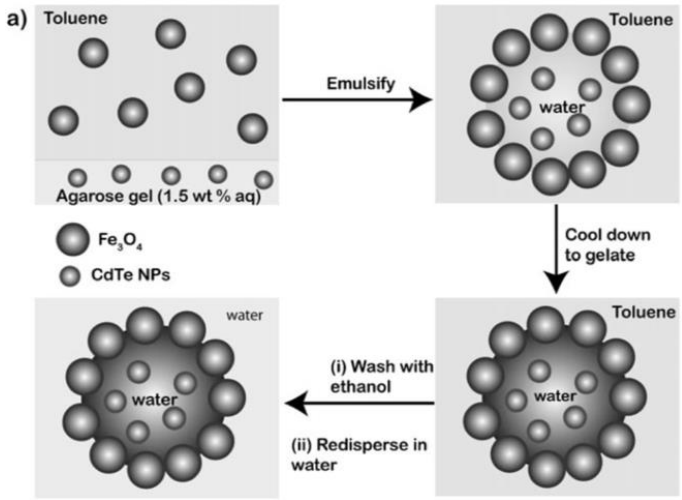

Figure 1.13. Schematic illustration of microcapsules fabricated based on self-assembly of Fe_3O_4 NPs at the toluene-water interface with encapsulated CdTe [3].

1.2. Click Chemistry

"Click chemistry" is a term which was coined by K. B. Sharpless in 2001 [41]. Following its disclosure, the popularity of click chemistry within the materials science increased because of the influential works of Hawker, Fréchet and Finn [42,43]. Click

chemistry offers products with high yield and selectivity under mild conditions, and only creates byproducts that can be removed without chromatography. These reactions are simple to conduct and can be performed in various solvents which are either benign (e.g. water) or easily removable. Additionally, these chemical reactions are in general tolerant towards the presence of numerous functional groups [44].

Click chemistry utilize pairs of complementary functional groups that quickly and selectively react with each other under mild conditions. These reactions can be divided into two separate groups; copper (I) catalyzed and copper-free. The Cu(I) catalyzed alkyne-azide cycloaddition (CuAAC) reaction requires Cu(I) ions, whereas the other reaction types includes the copper-free strain-promoted azide-alkyne click chemistry reaction (SPAAC), and the tetrazine-alkene ligation that can proceed without any metal catalysis [45]. The most widespread click chemistry reaction type is the reaction which occurs via the Cu^{I} catalysis because of easy availability of reactants. The Cu^{I}-catalyzed Huisgen reaction involves the 1,3-dipolar cycloaddition of azides and alkynes to yield a highly stable triazole compound (Figure 1.14). Because terminal alkynes are almost unreactive towards azide, the efficiency of a CuAAC reaction strongly relies on the presence of a metal catalyst like Cu in the +1 oxidation state. Although, various Cu(I) sources are available, *in situ* generation of Cu(I) through the combination of $CuSO_4$, a Cu(II) salt with sodium ascorbate as a reducing reagent is the one that is used extensively.

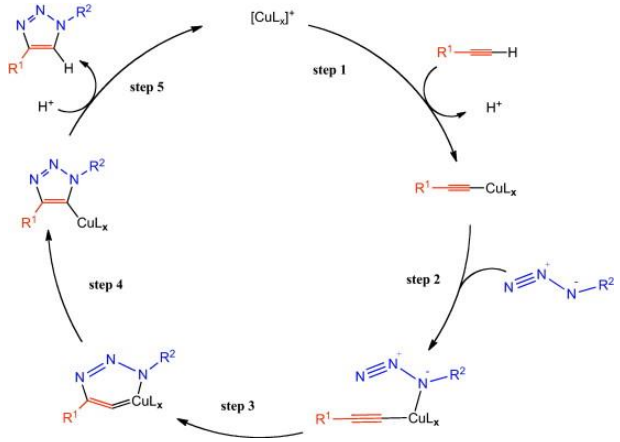

Figure 1.14. Mechanism of copper catalyzed Huisgen 1,3-dipolar cycloaddition [46].

In the presence of Cu(I), this reaction yields only 1,4-substituted products which makes this a highly regio-specific transformation. In general, the reaction proceeds at efficiently at room temperature. The reaction can be carried out in different solvents including water and also works over a wide range of pH. The Cu-catalyzed version of the reaction proceeds about 10^7 times faster than the Cu(I) free version. All of these characteristics of the Huisgen cycloaddition reaction have made it a popular transformation. Also, azides and terminal alkynes are easy to synthesize and have long-term stability under standard conditions [47]. In recent years, several studies have utilized this azide-alkyne click reaction for functionalization of NPs [48] and carbon nanotubes [49].

1.3. Thiol-Disufide Bond Exchange Systems

A disulfide bond (-S-S-) is a covalent linkage which takes place in the end of the oxidation of two sulfhydryl (SH) groups of cysteine or other -SH containing materials. Two different features make this bond useful to creating stimuli-responsive systems: its reversibility through redox-reaction, as well as its relative stability in plasma [50]. A disulfide bond can be easily cleaved by using thiol compounds, such as glutathione (GSH) and dithiothreitol (DTT) (Figure 1.15).

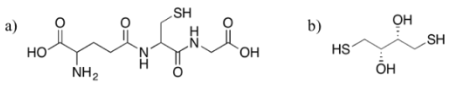

Figure 1.15. Chemical structures of (a) glutathione (GSH) and (b) dithiothreitol (DTT).

GSH and DTT have different efficiency on cleavage of disulfide bond. Since GSH has only one thiol group whereas DTT has two thiol groups that can allow intra-molecular reaction, DTT is more efficient in terms of its ability to cleave disulfide groups (Figure 1.16).

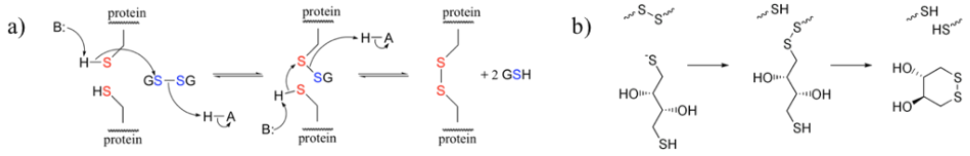

Figure 1.16. Mechanism of disulfide bond cleavage with (a) GSH and (b) DTT.

Cleavage of disulfide bonds through GSH is attractive since GSH is a protein that is naturally produced in human body. Importantly, the concentrations of intracellular and extracellular GSH are significantly different from each other. While inside the cells GSH amount is around 1-10mM, outside cells common fluids such as plasma contain 2-3 fold higher GSH [50,51].Because of this difference in concentration, a disulfide bond is stable under physiological circumstances both in the circulation and extracellular environment. However, it can be quickly cleaved in a highly reductive environment inside cells. This process leads to controlled intracellular release [52]. Likewise, it has been reported that the GSH concentration in tumor cells is several times higher than that in normal cells which act as a key role in the development of redox-responsive controlled drug delivery systems that are based on disulfide cleavage as a trigger [53].

Stimuli-responsive degradation of polymeric materials using disulfide-cleavage has been widely used. For example, Chen and coworkers synthesized a hyperbranched disulfide bonds containing poly (ester triazole)s via Cu(I) catalyzed alkyne-azide cycloaddition reaction [54]. The disulfide bonds on backbones enable the hyper branched poly (ester triazole)s with a reduction-cleavable feature in the presence of DTT. It was demonstrated that the hyperbranched polymer breaks down into smaller size polymeric blocks through cleavage of disulfide bonds in the presence of DTT (Figure 1.17).

Figure 1.17. The disulfide-based degradation of hyper branched poly (ester triazole)s induced by DTT [54].

2. AIM OF THE STUDY

The fabrication of colloidal microcapsules is very popular recently and it is developing day by day. Not only crosslinking provides a more stable and robust microcapsule but also reversibility of these systems are more useful to utilize them for lots of areas. The essential aim of my study is to form colloidal microcapsules via covalent interaction at the liquid liquid interface of water in oil emulsion. In order to form microcapsules, iron oxide nanoparticles were used and they were coated with convenient surfactant for click reaction. The reversibility of a cross-linked system is provided by disulfide bond system. With all these properties, fabricated microcapsules are suitable to be used as a carrier for any kind of molecule such as a drug and biomolecules (Figure 2.1).

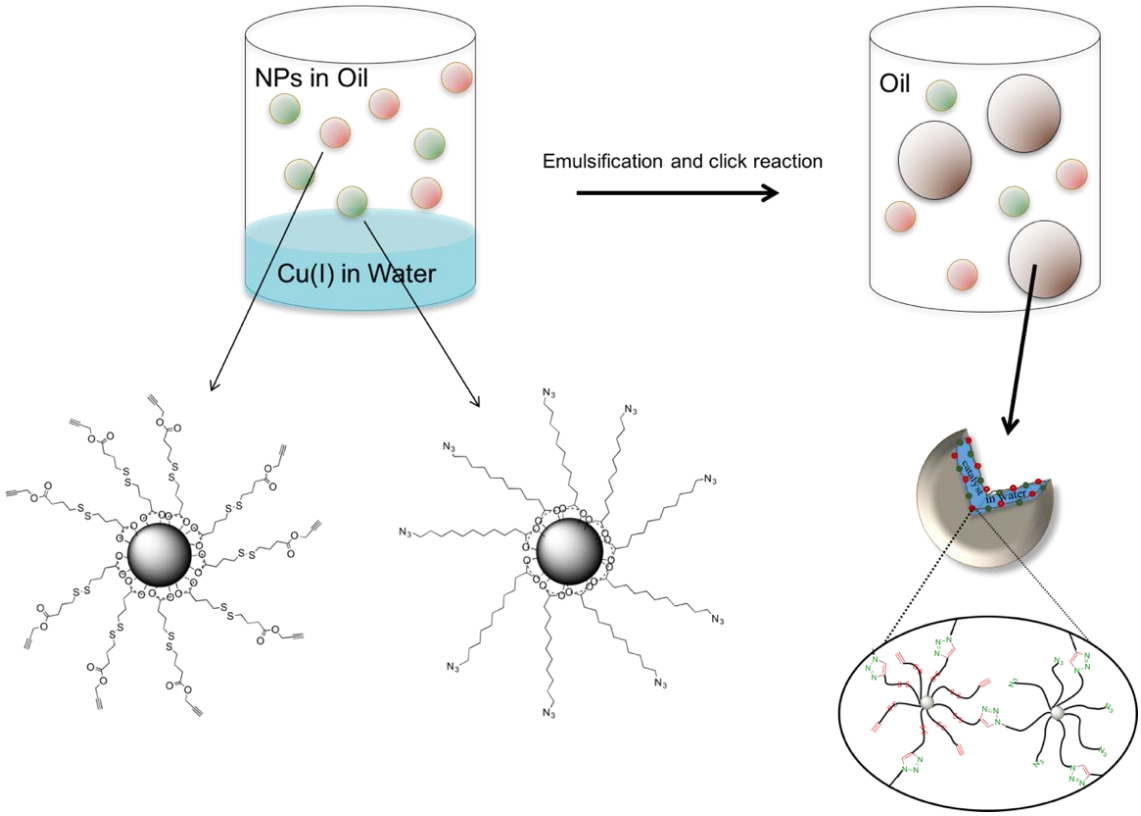

Figure 2.1. Cleavable microcapsules formation via click reaction.

3. EXPERIMENTAL

3.1. Methods and Materials

All chemicals used in experiments were received from producers (Aldrich, Alfa Aesar, Merck). Dry solvent (CH_2Cl_2) was supplied from ScimatCo Purification System. Column Chromatography was carried out using silicagel-60 (43-60 nm). For the thin layer chromatography, silica gel plates (Kiesel gel F254, 0.2 mm, Merck) were used. Infrared Spectroscopy was checked on Thermo Scientific Nicolet 380 FTIR spectrophotometer. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), optical microscopy and fluorescence microscopy were used to monitor microcapsules. ^1H Nuclear Magnetic Resonance (NMR) (operating at 400 MHz) was recorded and $CDCl_3$ was used as solvent at the Bogazici University.

3.2. Synthesis of Iron Oxide Nanoparticles

Oleic acid coated iron oxide nanoparticles were synthesized according to reported procedure [55].

3.3. Synthesis of Linkers

3.3.1. Synthesis of Disulfide Bond and Alkyne Containing Linker

4.4-dithiodibutyric acid (2g, 8.39 mmol), 4-dimethylaminopyridine (DMAP) (0.07g, 0.56 mmol) and N.N'-dicyclohexylcarbodiimide (DCC) (1.27g, 6.15 mmol) were taken into a round bottom flask and dissolved in dry dichloromethane (DCM) (25 ml). Then, propargyl alcohol (0.156g, 2.8 mmol) was added to the solution. The mixture was purged with nitrogen (N_2) for 10 min. After stirred for 20 h at room temperature under N_2, the solution was taken. For the purification, it was filtered through sintered glass and washed with ethyl acetate (EtOAC). Then, the mixture was extracted with H_2O and dried over sodium sulfate (Na_2SO_4). The solvent was removed

by rotary evaporation and the product was purified by column chromatography on a silica gel column. 1H NMR (400 MHz, $CDCl_3$) δ 4.67 (d, J = 1.7 Hz, 3H), 2.71 (td, J = 7.0, 3.9 Hz, 6H), 2.53 – 2.44 (m, 7H), 2.14 – 1.93 (m, 6H).

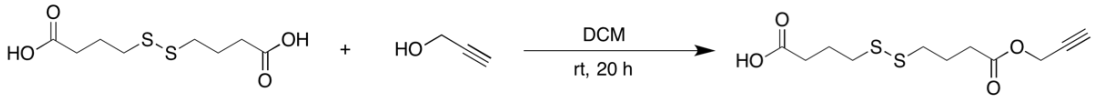

Figure 3.1. Synthesis of linker containing disulfide bond.

3.3.2. Synthesis of Azide Containing Linker

11-bromoundecanoic acid (1g, 3.77 mmol), sodium azide (NaN_3) (2.45g, 37.7 mmol) and potassium iodide (KI) (0.312g, 1.88 mmol) were dissolved in dimethyl sulfoxide (DMSO) (60 ml). The mixture was heated up to 80 ^oC and stirred for 2 days. After 2 days and it cooled down to room temperature, water (50 ml) was added to reaction mixture. First, it was extracted with EtOAC. Then, the organic phase was washed with brine solution. After it was dried over Na_2SO_4 and the solvent was removed by rotary evaporation, the final product was obtained. 1H NMR (400 MHz, $CDCl_3$) δ 3.23 (t, J = 6.9 Hz, 1H), 2.33 (t, J = 7.5 Hz, 1H), 1.67 – 1.52 (m, 2H), 1.40 – 1.16 (m, 6H).

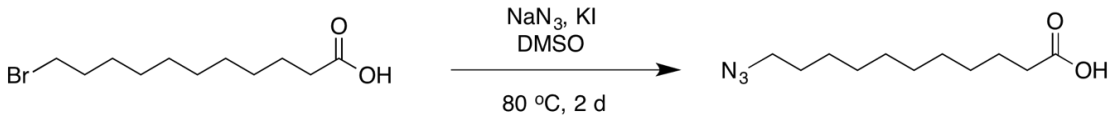

Figure 3.2. Synthesis of azide containing linker.

3.4. Place Exchange Reactions with Iron Oxide NPs

3.4.1. Place Exchange Reaction: Disulfide Bond Containing Alkyne Functionalized Iron Oxide NPs

Oleic acid coated iron oxide nanoparticles (40mg) and disulfide bond and alkyne containing linker (200mg) are dissolved in 5ml DCM and hexane (9:1). The

solution was purged with nitrogen and it was waited for 2 days at 50 ^{o}C. In order to purify, nanoparticles were precipitated in ethanol (EtOH), isolated using centrifugation and redispersed in DCM. This procedure was repeated until all unbound linker molecules were removed. Purified alkyne functionalized nanoparticles were kept in DCM.

3.4.2. Place Exchange Reaction: Azide Functionalized Iron Oxide NPs

Oleic acid coated iron oxide nanoparticles (40mg) and azide containing linker (200mg) are dissolved in DCM (5ml) and hexane (9:1). The solution was purged with nitrogen. Then, it was stirred for 2 days at 50 ^{o}C. To purify nanoparticles, they were precipitated in ethanol (EtOH) and isolated via centrifugation and redispersed in DCM. This procedure repeated until all unbound linker molecules were removed. Purified alkyne functionalized nanoparticles were dispersed in DCM for further usage.

3.4.3. Place Exchange Reaction: Alkyne Functionalized Iron Oxide NPs

Oleic acid coated iron oxide nanoparticles (40mg) and alkyne containing linker (200mg) are dissolved in DCM (5ml) and hexane (9:1). The solution was purged with nitrogen and it was stirred for 2 days at 50 ^{o}C. After the reaction, in order to purify, nanoparticles were precipitated in ethanol (EtOH) and isolated via centrifugation and redispersed in DCM. This procedure was repeated until all unbound linker molecules were removed. Purified alkyne functionalized nanoparticles were dispersed in DCM.

4. RESULTS AND DISCUSSIONS

Stable cleavable colloidosomes were fabricated by using Cu-catalyzed azide alkyne Huisgen cycloaddition between azide and alkyne groups at the interface of the water in oil emulsion. Two linker molecules one of which includes azide group and the other have both disulfide bond and alkyne group were synthesized. Then, azide and alkyne functionalized iron oxide nanoparticles were synthesized via place exchange reactions. Since one of the linkers has disulfide bond, microcapsules containing this group have sensitivity to reducing agents such as DTT. After forming microcapsules, they were monitored under optical microscope. Then a fluorescence dye was encapsulated into microcapsules and their stability in medium with DTT was investigated by using fluorescence microscope.

4.1. Fabrication of Microcapsules

4.1.1. Synthesis of Iron Oxide Nanoparticles

Oleic acid stabilized iron oxide nanoparticles were synthesized with the procedure described before [65]. The IR spectrum of NPs showed peaks coming from CH_2at 2915 cm^{-1} and 2846 cm^{-1}, respectively. Besides, characteristic Fe-O peak can be seen at 549 cm^{-1} which demonstrates forming of iron oxide nanoparticles (Figure 4.1).

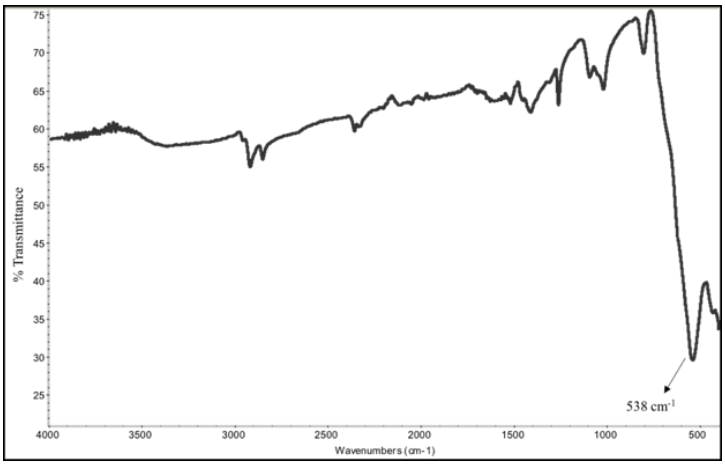

Figure 4.1. FT-IR spectrum of oleic acid coated iron oxide NPs.

They were characterized by FT-IR spectroscopy and transmission electron microscopy (TEM) (Figure 4.2).

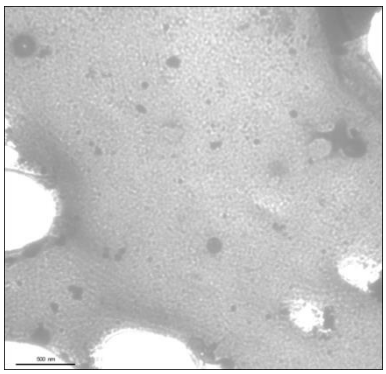

Figure 4.2. TEM image of oleic acid coated iron oxide NPs.

4.1.2. Functionalization of Iron Oxide NPs with Alkyne Containing Linker

Fabricated iron oxide NPs were then functionalized with alkyne group and disulfide bond containing linker by using place exchange reaction (Figure 4.3).

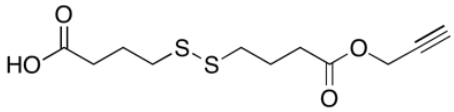

Figure 4.3. Structure of alkyne and disulfide bond containing linker.

NPs were coated with alkyne and disulfide bond containing linker via place exchange reaction (Figure 4.4).

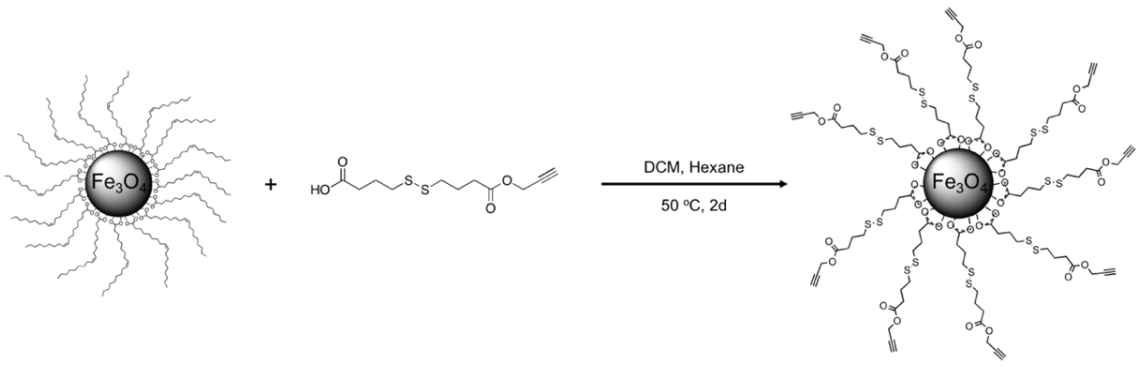

Figure 4.4. Synthesis of disulfide bond containing iron oxide NPs.

Alkyne functionalized NPs were identified by FT-IR spectroscopy. IR spectrum showed CH_2 peaks at 2925 cm^{-1} and 2854 cm^{-1}(Figure 4.5).

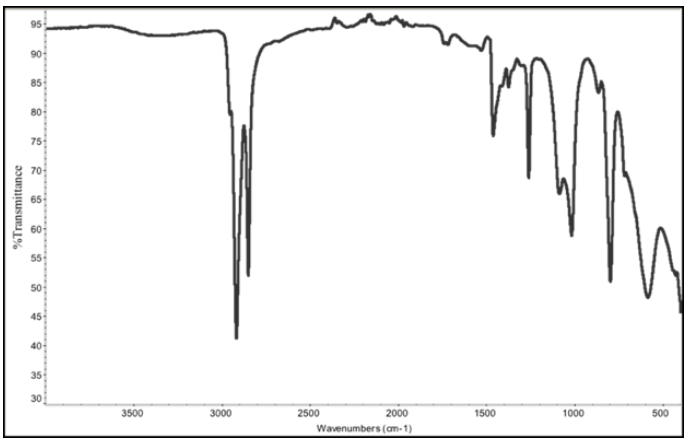

Figure 4.5. FT-IR spectrum of alkyne functionalized iron oxide NPs.

4.1.3. Functionalization of Iron Oxide NPs with Azide Containing Linker

Oleic acid coated iron oxide NPs were functionalized with linker containing azide group via place exchange reaction (Figure 4.6).

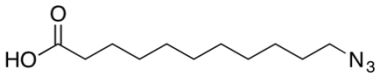

Figure 4.6. Structure of azide containing linker.

By a place exchange reaction, NPs were coated with azide bearing linker (Figure 4.7).

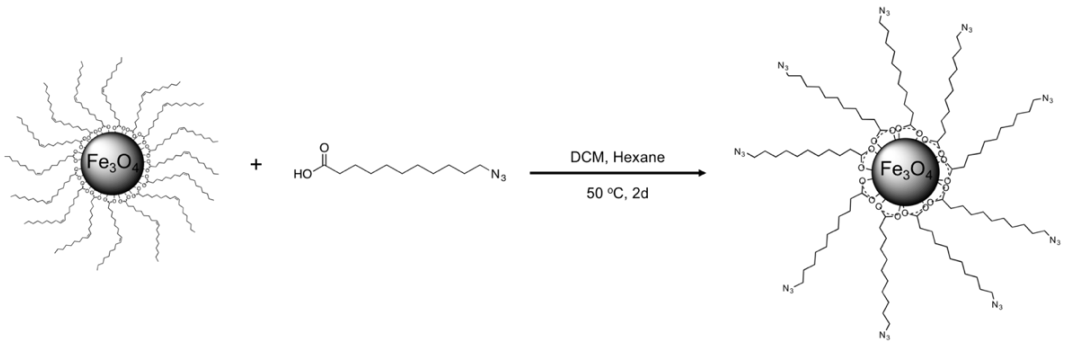

Figure 4.7. Synthesis of azide functionalized iron oxide nanoparticles.

Azide functionalized NPs were characterized by FT-IR spectroscopy. IR spectrum showed CH_2 asymmetric and symmetric peaks at 2920 cm^{-1} and 2851 cm^{-1}. Also the peak belonging to azide located at 2115 cm^{-1} (Figure 4.8).

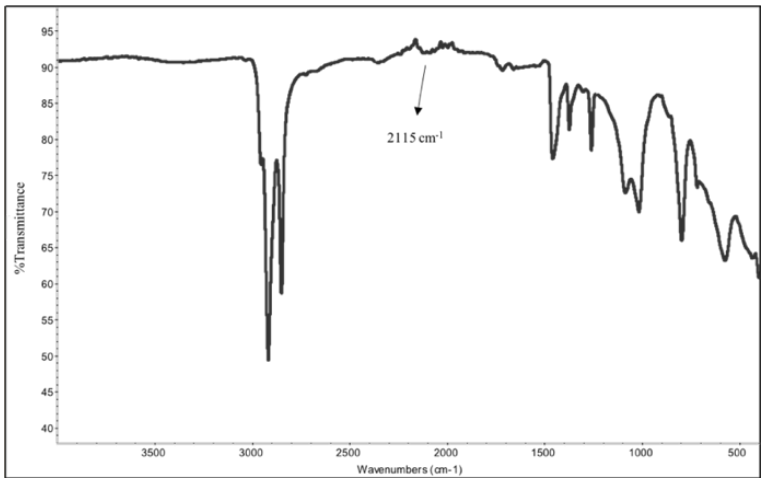

Figure 4.8. FT-IR spectrum of azide functionalized iron oxide NPs.

4.1.4. Functionalization of Iron Oxide NPs with 10 Undecynoic Acid

As a control group, iron oxide NPs functionalized with oleic acid were coated with commercial linker containing only alkyne group via place exchange reaction (Figure 4.9).

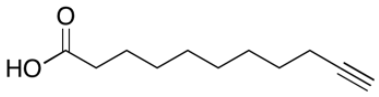

Figure 4.9. Structure of linker containing alkyne group.

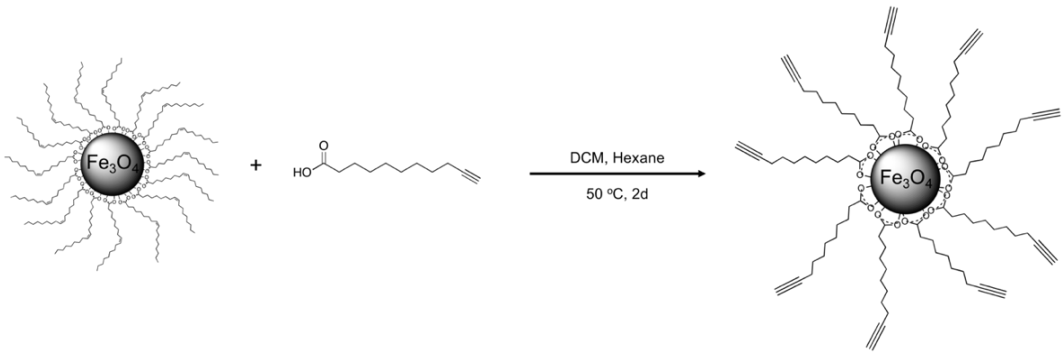

Figure 4.10. Synthesis of alkyne functionalized iron oxide NPs.

By a place exchange reaction, NPs were coated with only alkyne group bearing linker (Figure 4.10).

After place exchange reaction, alkyne functionalized NPs were characterized by FT-IR spectroscopy. IR spectrum showed CH_2 peaks at 2920 cm^{-1} and 2851 cm^{-1}. Also the alkyne peak can be seen at around 3300 cm^{-1} and peak belonging to iron oxide NPs at around 500 cm^{-1} (Figure 4.11).

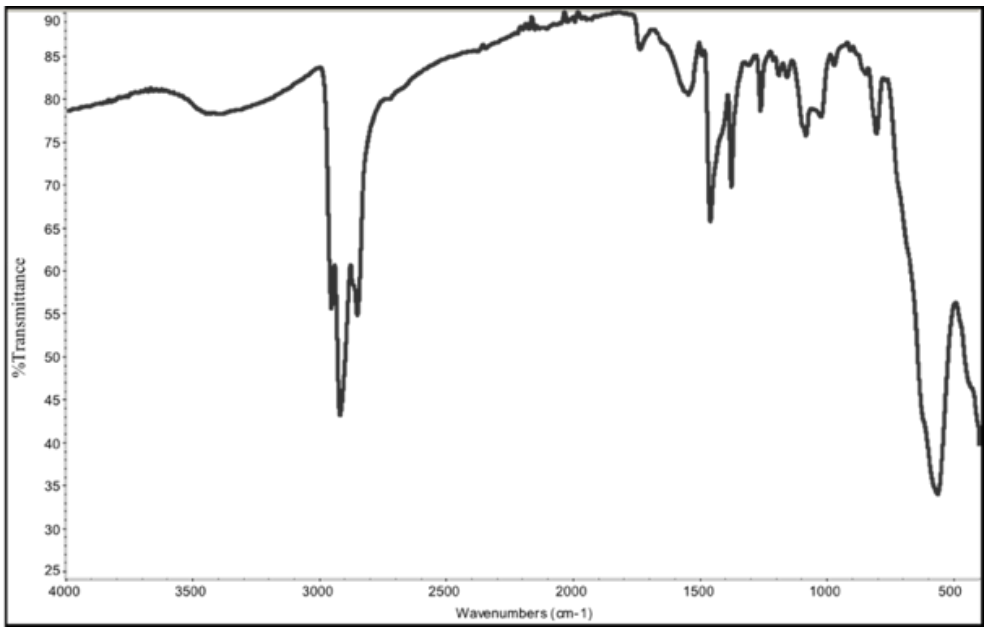

Figure 4.11. FT-IR spectrum of alkyne functionalized iron oxide NPs.

4.1.5. Synthesis of Microcapsules

Firstly, concentration of alkyne and azide functionalized NPs solutions in DCM was set to 10 mg/mL. Then, alkyne and azide in toluene were taken in a vial. Cu(I) catalyst was prepared in a separate vial using $CuSO_4.5H_2O$ (1.0 eq) and NaAsc (10.0 eq) in NaCl solution (100 μl, 10mM) and it was put the NPs mixture quickly and the vial was vigorously shaken by hand for 30 seconds, followed by a wait period of 30 minutes. After 30 min, first the oil phase (toluene) was changed with fresh toluene carefully with the help of a micropipette and then the water phase was cleaned by changing with fresh water. Finally, stable microcapsules were obtained at the bottom of the vial.

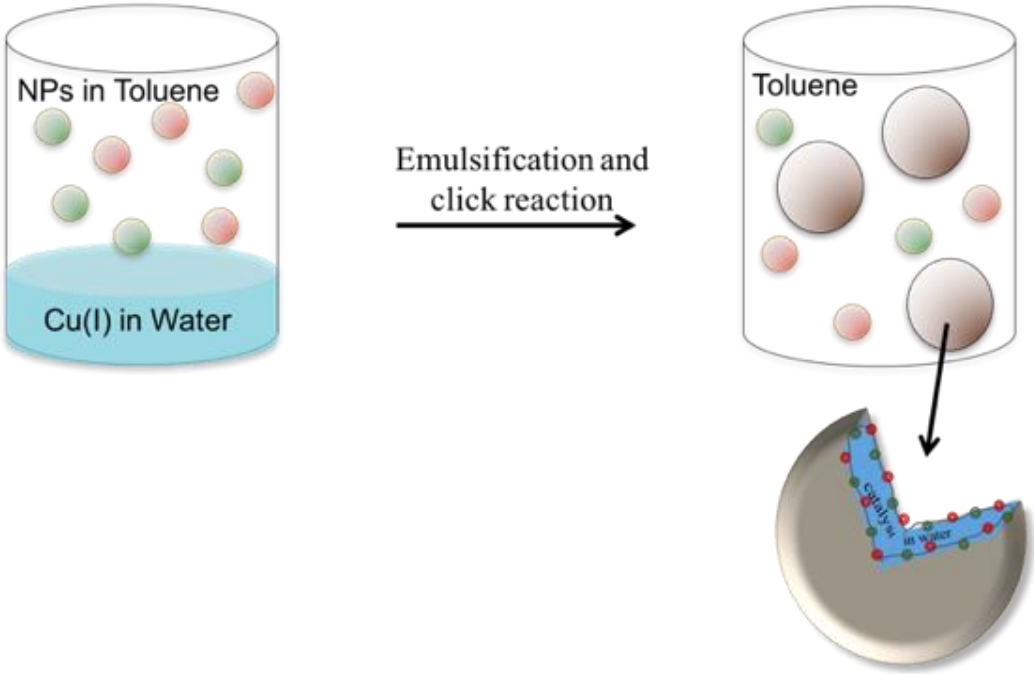

Figure 4.12. Schematic illustration of formation of microcapsules.

Two types of stable microcapsules were fabricated by using click reaction between alkyne and azide functionalized iron oxide NPs. One of them used the disulfide bond containing NPs and the other used NPs without the disulfide bond (Figure 4.13). In appearance, visually these MCs looked the same.

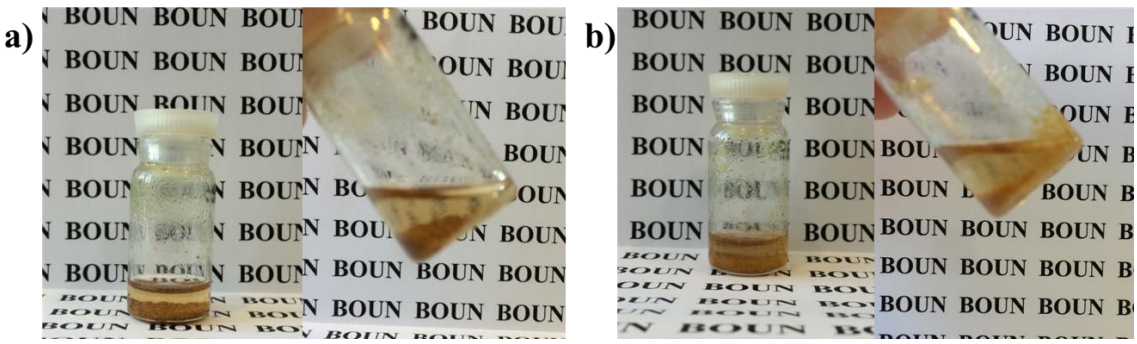

Figure 4.13. MCs a) with and b) without disulfide bond.

Besides, they were visualized under optical microscope which revealed that they have spherical shape with size ranging between 30-50 μm (Figure 4.14).

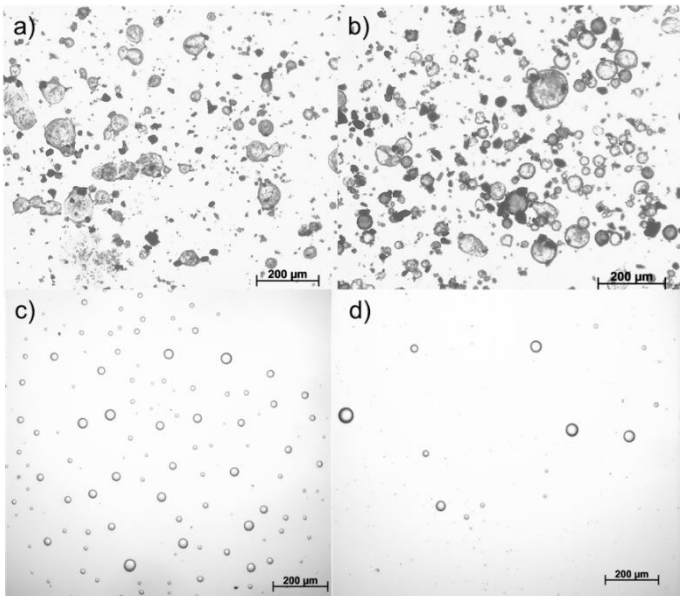

Figure 4.14. Optical images of MCs with and without disulfide bond before (a, b) and after wash (c, d), respectively.

Microcapsules were stable in water solution for at least 1 week. In order to show this, they were checked after 1 day while they were wet and dried (Figure 4.15)

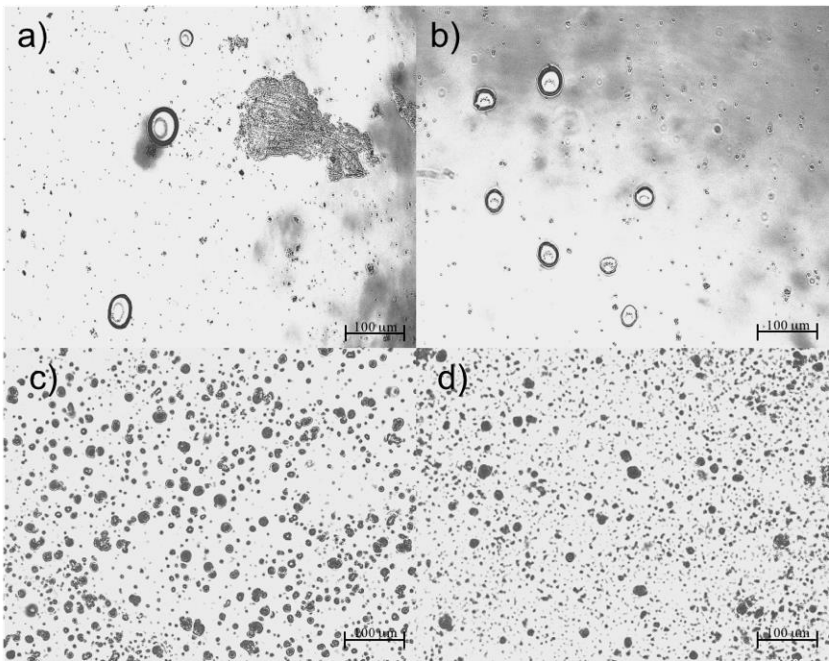

Figure 4.15. Optical micrographs 1 day after, MCs with and without disulfide bond (a, b) and after dried (c, d), respectively.

Also after 1 week, they were checked again (Figure 4.16).

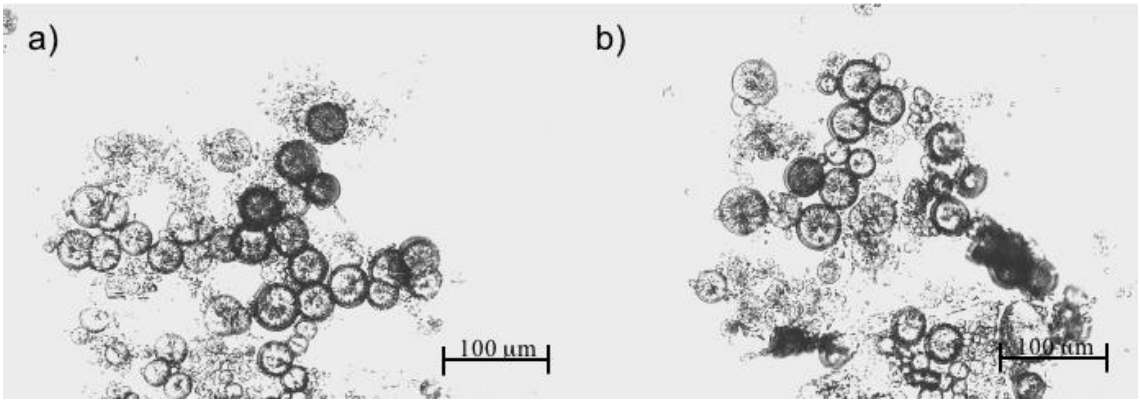

Figure 4.16. Optical micrographs 1 week after, MCs with and without disulfide bond (a, b), respectively.

In addition to these, microcapsules were also visualized by scanning electron microscope (SEM). Their sizes were around 10-15 µm (Figure 4.17). Because microcapsules had water inside them and were dried before looking SEM, they lost the water inside upon drying and shrinkage was observed.

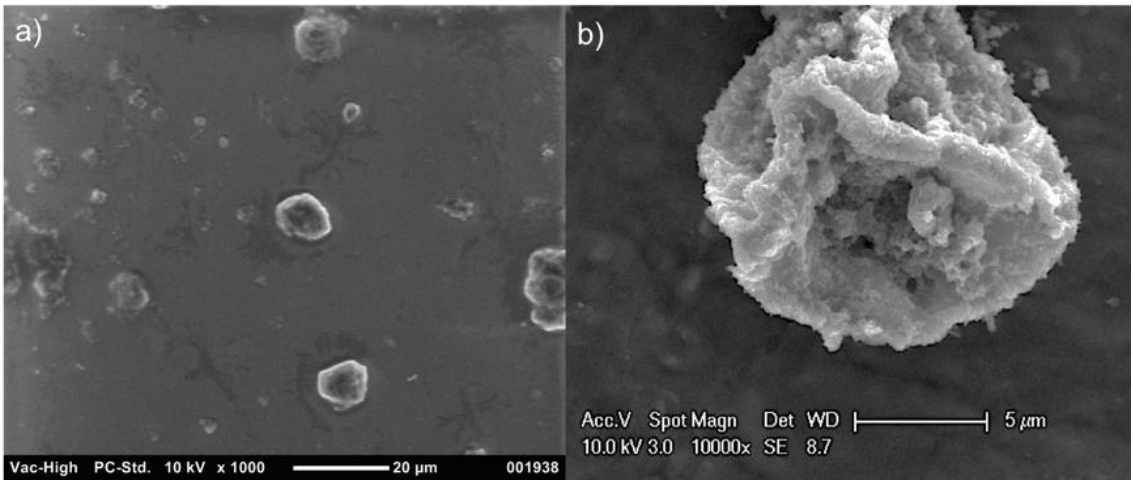

Figure 4.17. SEM images of dried MCs.

Iron oxide particles have magnetic properties as mentioned in the introduction part. Since there will be no change in this feature of particles during the click reaction, microcapsules which were made from iron oxide NPs will have the same magnetic features with iron oxide NPs (Figure 4.18).

Figure 4.18. Magnetic properties of MCs.

4.2. Encapsulation of Fluorescent Dye into the Microcapsules

Since microcapsules have water phase in them, it renders possible to encapsulation of any hydrophilic dye into the microcapsules. To prove that, fluorescein isothiocyanate (FITC) labeled dextran (dex-FITC) was used during fabrication of microcapsules. While forming microcapsules, dex-FITC solution (0.5 mg/ml H_2O) was added to water in toluene phase right before shaking. Then, encapsulation of hydrophilic fluorescent dye was visualized under fluorescence microscope (Figure 4.19).

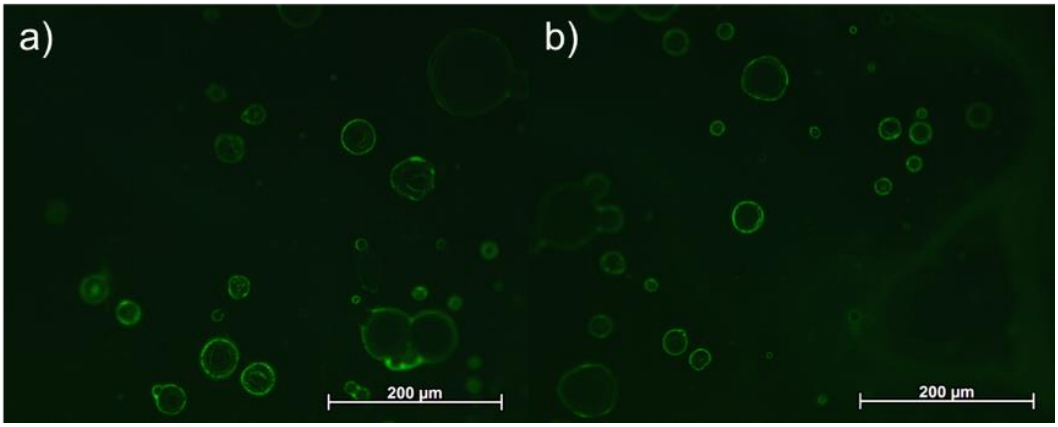

Figure 4.19. MCs containing dex-FITC with (a) and without (b) disulfide bond.

4.3. Cleavage of Redox Responsive Microcapsules with DTT

After microcapsules were fabricated and washed with fresh water, dithiothreitol (DTT) solution was added to them. Cleavage of disulfide bonds on microcapsules was monitored approximately 15 minutes, under optical microscope. It is obvious that when microcapsules including disulfide bond were cleaved, most of the microcapsules without disulfide bond were stable during same time.

Nevertheless, some microcapsules which do not include disulfide bond had disruption since the microscope light can lead to an increase of the temperature in environment and evaporation the water inside microcapsules. But still these microcapsules are negligible from the standpoint of staying stable.

On the other hand, microcapsules with disulfide bond showed a degradation when DTT was added. Besides, the cleavage time of these microcapsules depended on the concentration of both DTT and microcapsule solution.

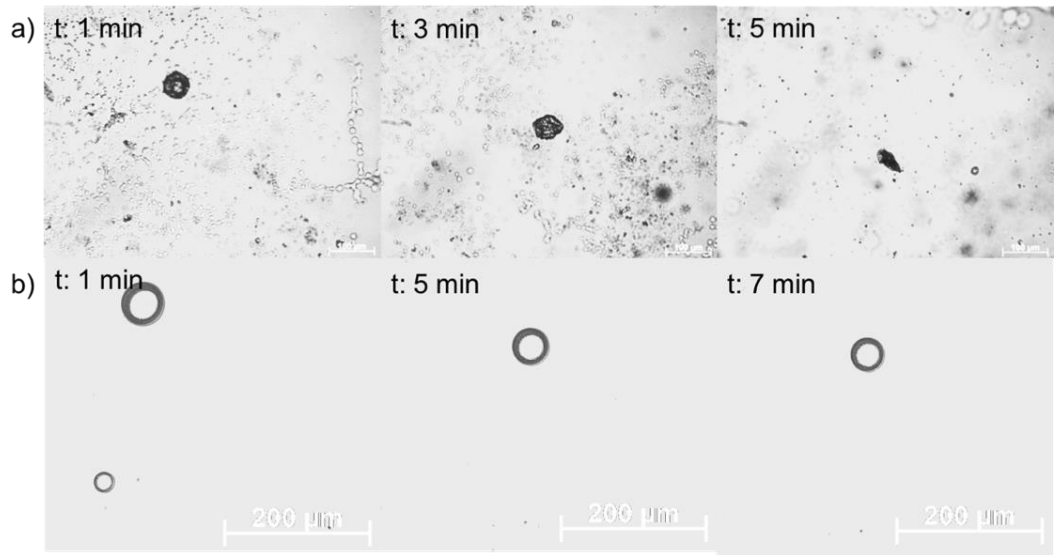

Figure 4.20. Cleavage of MCs with 200 mM DTT a) with and b) without disulfide bond.

Firstly, cleavage of microcapsules was studied with 200 mM DTT solution which was much more than microcapsules which need. Microcapsules containing

disulfide bond were almost destructed in 3 min and after 5 min the spherical structure of microcapsule was completely gone. However, microcapsules without disulfide bond were stable for 7 min and more (Figure 4.20).

After the study with 200 mM DTT, the same experiment was done with 25 mM DTT. Microcapsules with disulfide bond were cleaved in 7 min (Figure 4.21). Although the concentration of DTT was completely different, the cleavage occurred almost the same time.

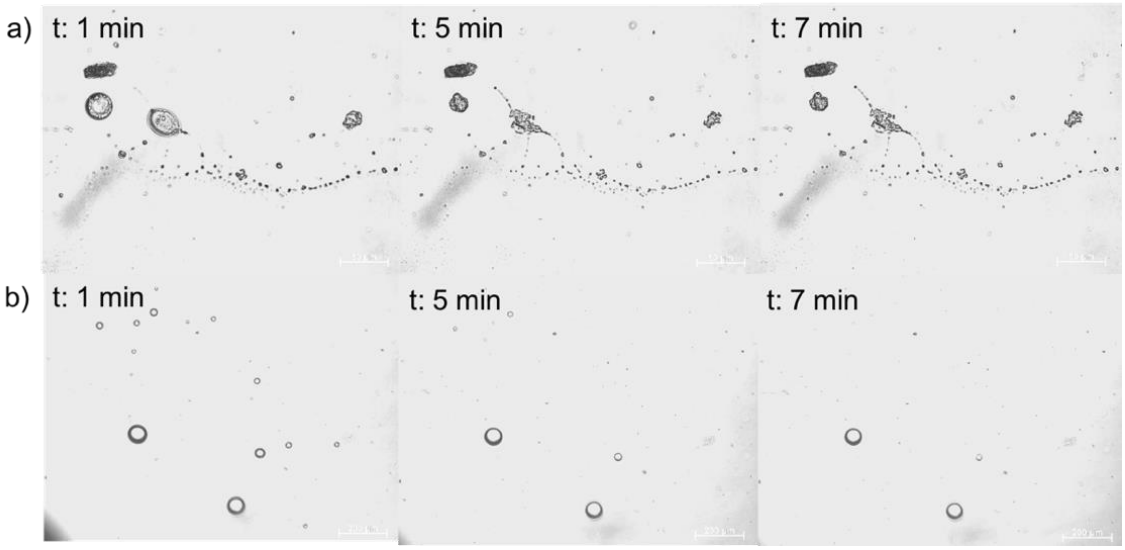

Figure 4.21. Cleavage of MCs with 25 mM DTT a) with and b) without disulfide bond.

4.4. Dye Release from Microcapsules by Using DTT Cleavage

Initially we encapsulated the dye into the microcapsules in accordance with the procedure as mentioned before. The dye is FITC labeled dextran (mw: 500.000) and it shows green fluorescence. 15 μl dextran FITC solution was added to water phase during the formation of microcapsules.

For the release of FITC labelled dextran dye, after fabricated microcapsules including FITC labelled dextran, DTT solution was added to microcapsules in water and they were monitored under fluorescence microscope. Thus cleavage of disulfide

bonds was checked by the time and release of dye from microcapsules could be visualized as green color inside microcapsules (Figure 4.22).

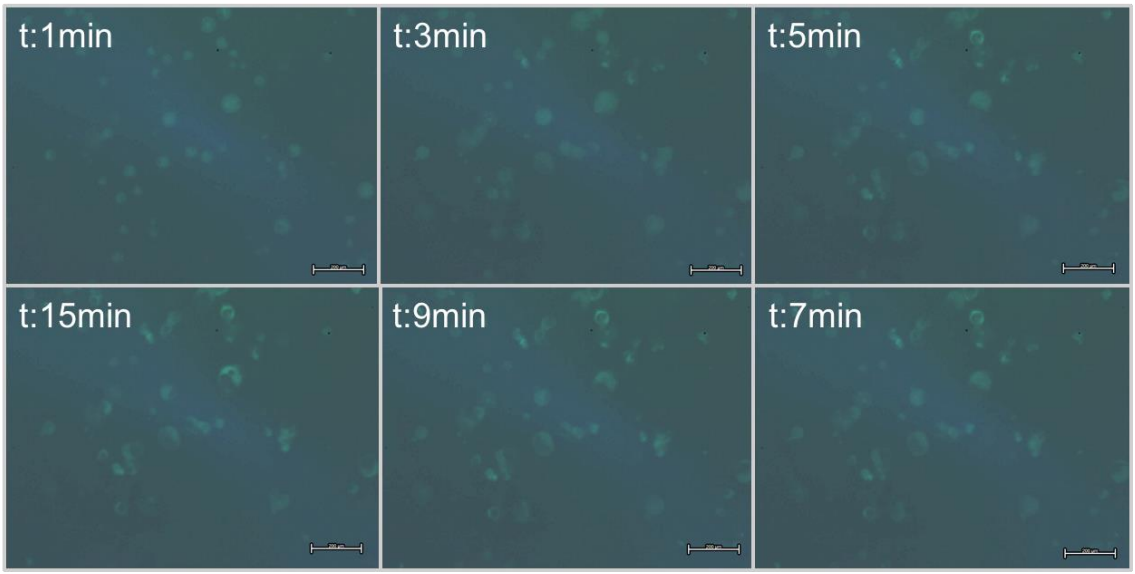

Figure 4.22. Release of dextran-FITC from MCs.

Release of dex-FITC from microcapsules was also followed by observing them under UV-irradiation. After microcapsule formation, MCs were taken in fresh water and DTT solution was added. In the beginning, microcapsules containing disulfide bond gathered on the surface of the water. After a few minutes, disulfide bonds were broken with DTT and dye was released. Thus, dye was dispersed all throughout the water and solution possessed a green fluorescence (Figure 4.23).

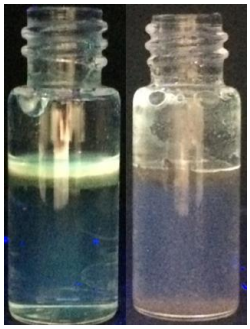

Figure 4.23. Release of dextran-FITC from MCs with disulfide bond.

Additionally, the left vial was a control experiment. While DTT was added to the right vial, only water with the same volume of DTT solution was added to left vial.

Finally, in the left vial, microcapsules stayed only on the water while microcapsules in the right vial dispersed.

Dye releasing form microcapsules experiment was repeated also for microcapsules without disulfide bond to prove that the dye released because of disulfide bond cleavage (Figure 4.24).

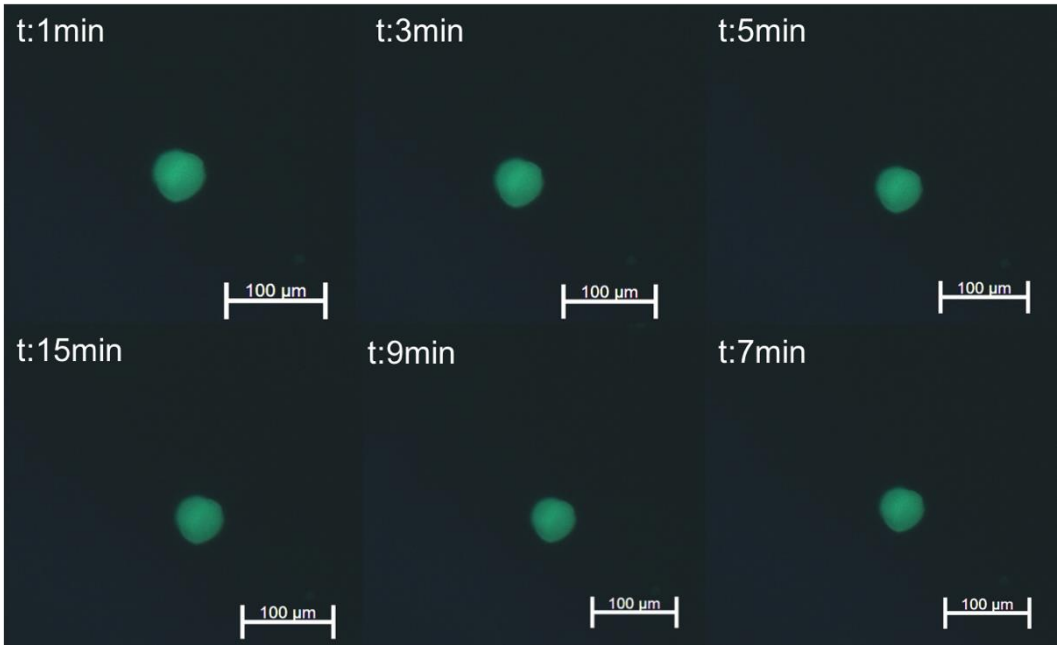

Figure 4.24. Release of dextran-FITC from MCs without disulfide bond.

Under the UV-irradiation, microcapsules without disulfide bond could be seen as clumped together at the air-water interface (Figure 4.25).

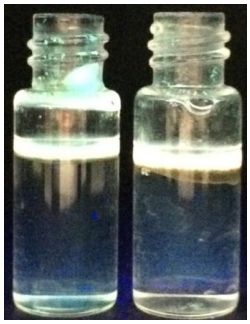

Figure 4.25. Lack of release of dextran-FITC from MCs without disulfide bond.

As the same with former experiment, the right vial had DTT solution and the left vial included only water with the same volume of DTT solution. This experiment was undertaken to probe the stability of MCs without disulfide bond in the presence of DTT.

5. CONCLUSION

Stable yet cleavable microcapsules from nanoparticles were obtained using their assembly and crosslinking at the oil-water interface. Interparticle ligands containing azide and alkyne groups were crosslinked using copper catalyzed alkyne-azide cycloaddition reaction. The alkyne ligand was designed to contain a disulfide unit that would be cleaved in presence of thiol containing molecules such as glutathione and dithiothreitol (DTT). First, nanoparticles containing alkyne and azide containing linkers were successfully synthesized. Using these ligands modified nanoparticles, water in toluene emulsion containing the nanoparticles was prepared and microcapsules were fabricated via click reaction between alkyne and azide groups. Fabricated microcapsules were at least stable up to 1 week. Microcapsules were used as a carrier for encapsulating biomolecules. In order to show that, a dextran polymer appended with a fluorescent dye was trapped within the microcapsules and its release was monitored using fluorescence microscope. It was shown that in the presence of an external thiol, no cleavage of capsule and no release was observed from microcapsules that did not possess a disulfide unit. Whereas microcapsules constructed using disulfide units were cleaved in the presence of thiols to release the encapsulated material.

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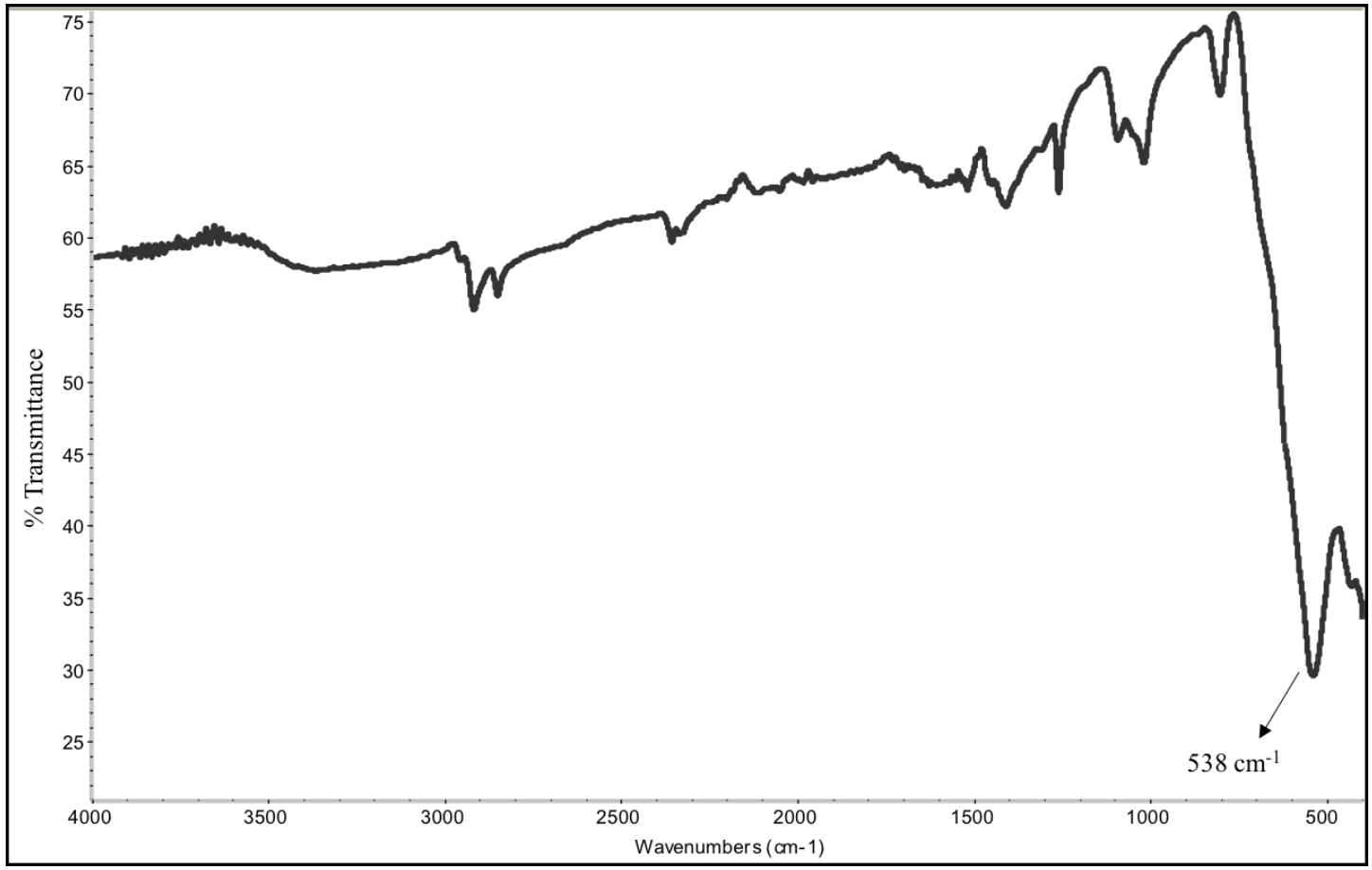

Figure A.1. FT-IR spectrum of oleic acid coated iron oxide nanoparticles.

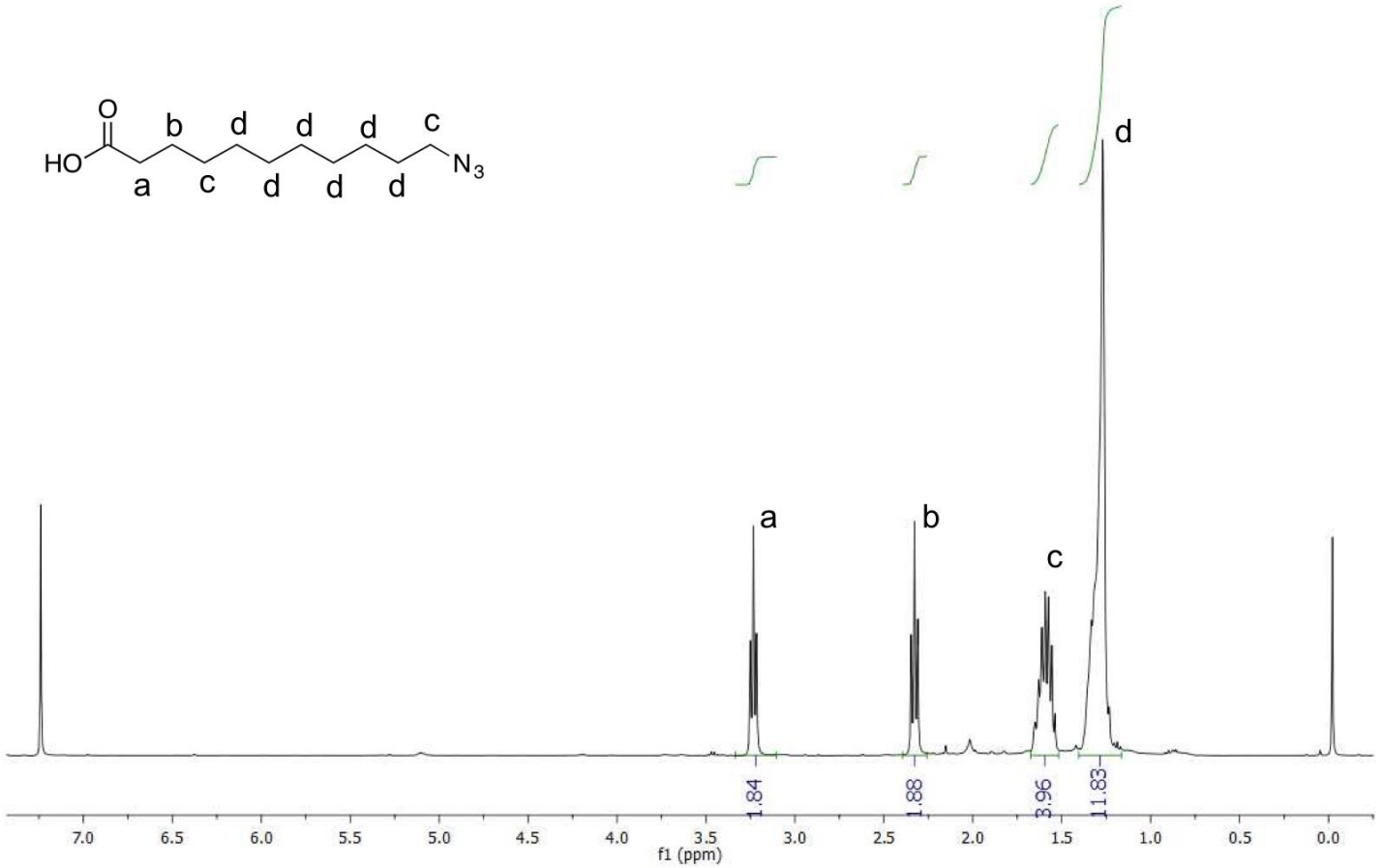

Figure A.2. ^{1}H NMR spectrum of linker bearing azide group.

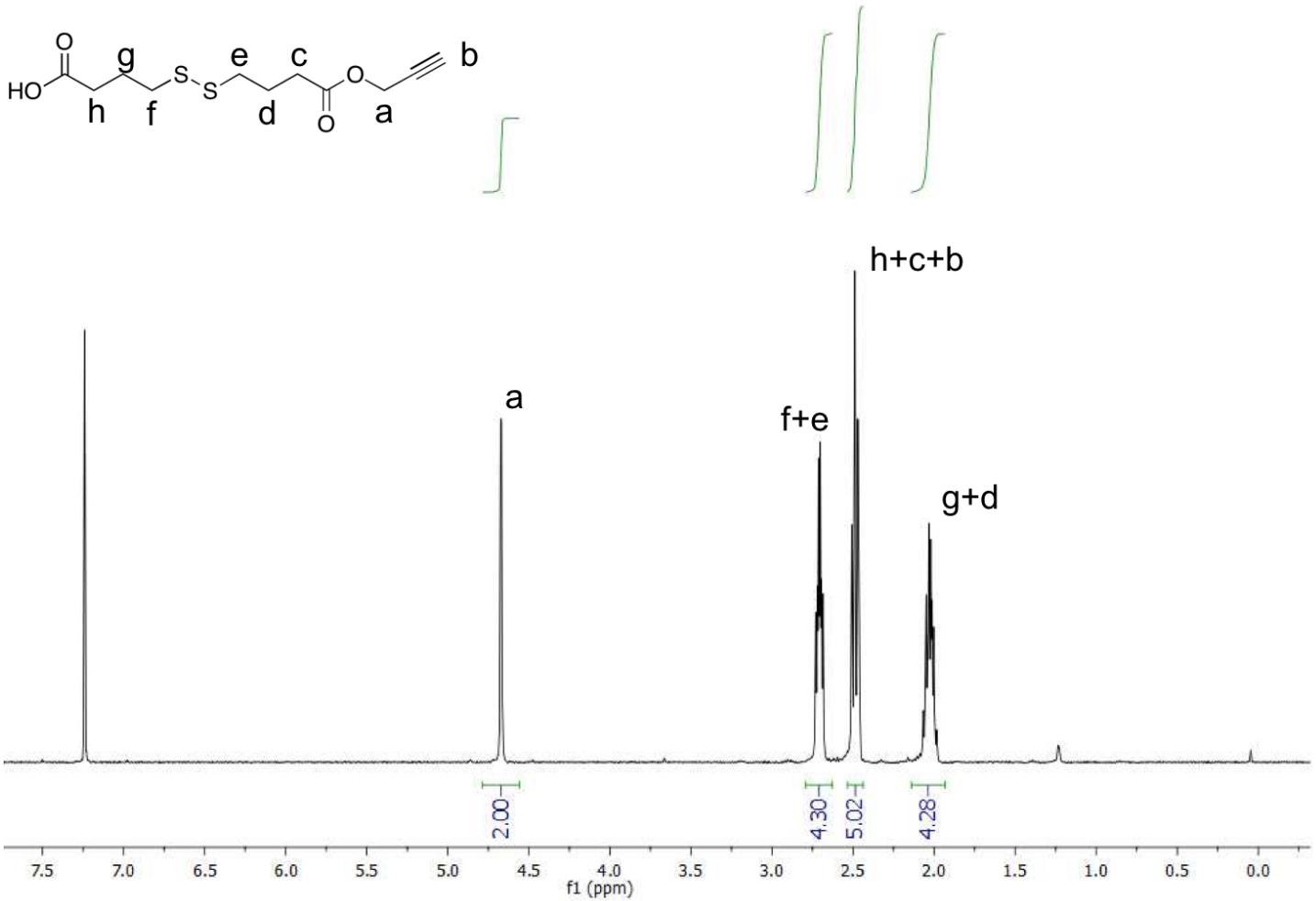

Figure A.3. H NMR spectrum of linker containing both disulfide bond and alkyne group.

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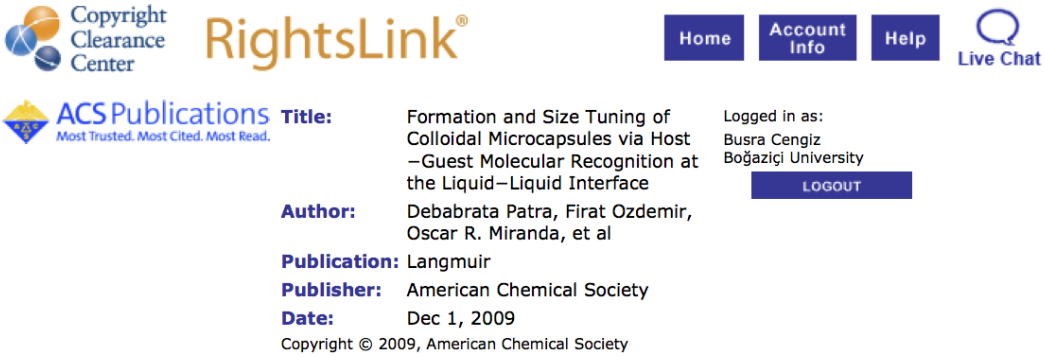

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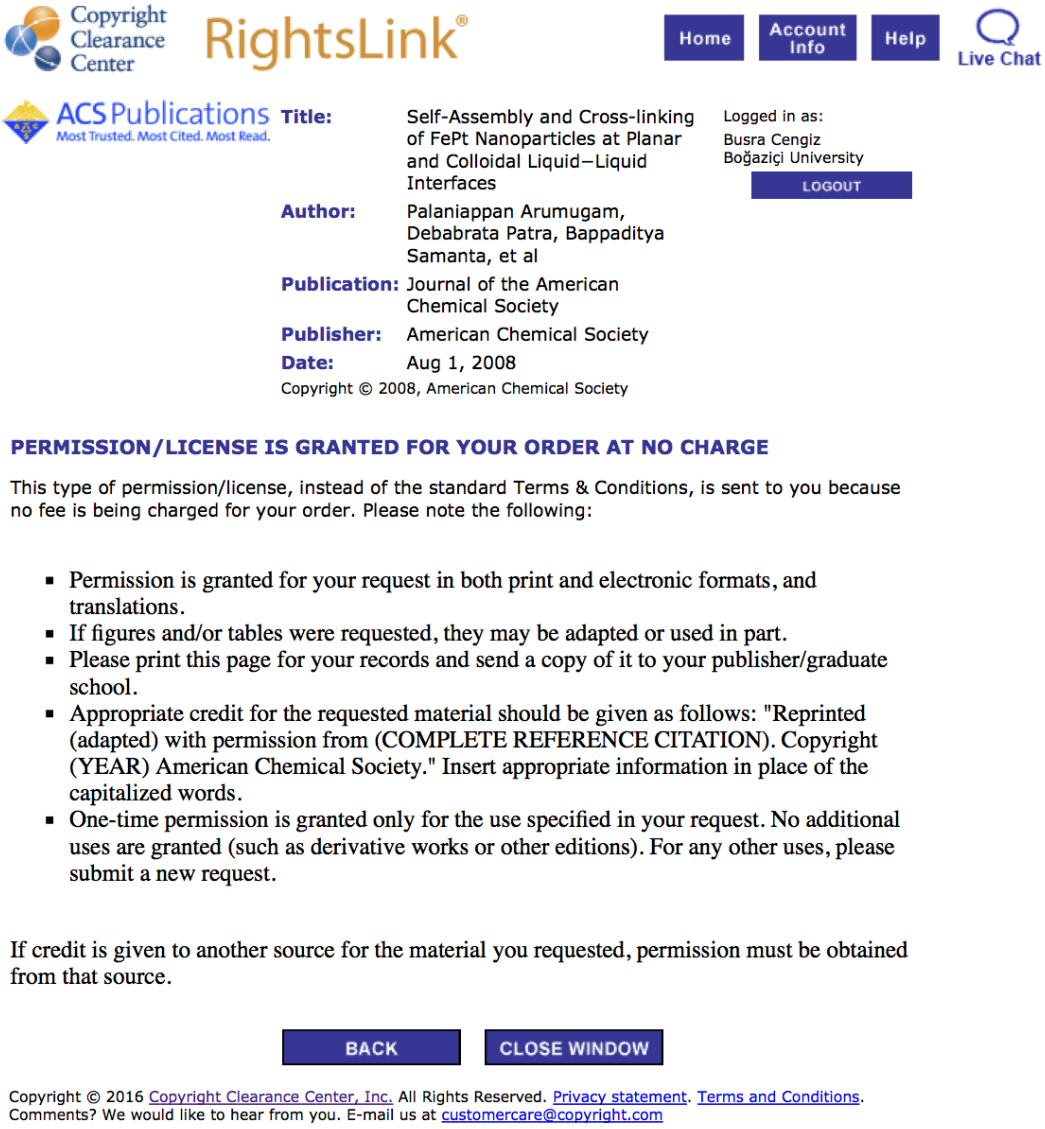

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