

Functional Magnetic Nanoparticles for Protein Delivery Applications

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Abstract

Iron oxide nanoparticles (IONPs) surface functionalised with thermo-responsive polymers present a potential strategy for biomolecule delivery and release. Here, the synthesis of thermo-responsive poly-*N*-isopropylmethacrylamide (PNIPMAM) coated IONPs is reported. The lower critical solution temperature (LCST) of the polymer-shell NP was tuned by using different chain length PNIPMAM shell (7.5 – 89 kDa) on the NP core (15.4 ± 2.1 nm IONPs). The LCST of all the core-shell nanostructures was above 37 °C, where the LCST of 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs was ~ 45 °C. These core-shell NPs were then screened for their magnetic heating behaviour where 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs showed maximum heating with a specific absorption rate (SAR) value of ~ 7.5 W/g (magnetic field strength = 28.7 mT, and frequency = 102.4 kHz). After LCST and magnetic heating characterization, these core-shell NPs were screened for the encapsulation/triggered release of a model protein apotransferrin (TRF). The protein release was observed in the presence of a competitor protein (RNaseB) where, 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs displayed a good TRF release profile (45 °C) with minimum protein leak at 37 °C. After the optimization of the core-shell nanostructure for TRF encapsulation/triggered release, effect of competitor protein properties (size and glycosylation) was studied on the triggered TRF release. Glycosylated protein competitor (RNaseB) released ~ 20 ng of the encapsulated TRF (~ 400 ng) compared with the similar size (~ 14 KDa) non-glycosylated RNaseA (~ 2 ng). Additionally, bigger glycosylated proteins than RNaseB were better competitors for the triggered TRF release (~ 170 ng, ovalbumin ~ 45 KDa). Serum was also tested as a source of competitor proteins for the magneto-thermal protein release. Magnetic heating, through a pulsed application resulted in a faster protein release as compared to the conventional heating of the protein loaded PNIPMAM @ IONPs. Hence the developed thermo-responsive core-shell NP could be a potential tool for the *in vivo* biomolecule delivery/temperature-sensitive release.

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List of accompanying material

Appendices are provided and referenced throughout this thesis as (**Appendix**). This includes standard curves for 6-nitrodopamine (NDA) and green fluorescent protein (GFP), polymer shell thickness calculations for polymer-coated nanoparticle, mesh size calculations for agar hydrogel, dynamic light scattering (DLS) data of iron oxide nanoparticles, representative Western blots for different protein encapsulation/release experiments, NDA-terminated poly(*N*-hydroxyethyl acrylamide) (NDA-PNHEA) synthesis characterisation spectra and the setup used to monitor magnetic heating and gel diffusion of the NPs.

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Declaration

I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, university. All sources are acknowledged as references.

The methodology for the apotransferrin (TRF) encapsulation/triggered release in the presence of a competitor (RNaseB) was developed by Matthew Walker. This procedure was later adapted for the protein loading/release described in this thesis, which was performed by the author (**Section 5.2.1**).

Chapter 1: Introduction

Magnetic iron oxide nanoparticles (IONPs) such as magnetite (Fe_3O_4) and maghemite ($\gamma\text{-Fe}_2\text{O}_3$), have found widespread use in the biomedical field owing to their unique and tuneable magnetic properties.¹ They have been used as contrast agents in magnetic resonance imaging,² biosensor materials;³ their use as drug delivery agents is under progressive intense development.⁴⁻⁵ Iron is involved in the human metabolic pathways which makes IONPs suitable for the *in vivo* applications.⁶ Upon the degradation of magnetite in the human body, a specialized protein ferritin is able to store the iron released in the ferric form (Fe^{3+}) and helps in recycling process of iron. It also limits the harmful reaction between ferrous iron (Fe^{2+}) and hydrogen peroxide known as Fenton process which generates highly damaging hydroxyl radicals.⁷⁻⁸ Most of the studies in the literature generally support the idea of low cytotoxicity of IONPs and revealed that the cytotoxic effects of magnetite are usually negligible⁸⁻⁹ or limited to very high doses ($> 0.5 \text{ mg/ml}$) or longer exposure ($> 120 \text{ h}$).¹⁰ In fact, the Food and Drug Administration (FDA) of the USA has approved iron oxides as contrast agent for magnetic resonance imaging (MRI).⁹

Most of the biomedical applications are largely based on the distinctive magnetic and other physicochemical properties of the NPs which strongly depend on the synthesis method.⁵. Because of their superparamagnetic behaviour, in an alternate magnetic field (AMF), IONPs exhibit a heating phenomenon known as magnetic hyperthermia (MH). The aim of our project was to utilise magnetic properties of polymer-coated IONPs for protein delivery applications, and therefore this chapter will first describe synthesis and characterisation of IONPs, followed by discussion of their magnetic properties with particular focus on magnetic hyperthermia as it is relevant to the proposed mode of action.

1.1. Synthesis of IONPs: General approaches and LaMer model

Magnetic IONPs can be obtained by various physical or chemical methods. The physical methods consist of top-down processes such as laser induced ablation of macroscopic targets of iron or iron oxides and mechanical milling of bulk iron oxide.^{11,12} Both physical approaches

have some limitations; laser ablation approach gives polycrystalline IONPs with wide size distributions and in mechanical milling, the degree of crystallinity of the product is reduced as compared to the starting material.^{11,12}

The chemical methods are bottom-up processes to synthesize IONPs from iron ions or molecular precursors. The chemical methods offer great advantage of controlling the composition, size, and shape to tune the desired properties by changing the synthesis conditions. The most described methods to synthesize uniformly sized IONPs, comprise alkaline co-precipitation of aqueous ferrous and ferric salts, hydrothermal treatment, thermal decomposition of organometallic iron complexes, and polyol process. In all synthesis methods, the mechanism of the formation of IONPs from atomic precursors can be described by LaMer model (**Fig. 1.1**).^{13,14} According to this model, three processes take place during the synthesis of IONPs: nucleation, crystal growth, and Ostwald ripening. The basic idea of LaMer model for the NP formation is to separate nucleation and crystal growth, meaning that a burst of nucleation occurs at the early synthesis stage (homogeneous growth), followed by crystal growth through diffusion of the reactants to the nuclei (heterogeneous nucleation). From the soluble precursors, molecular precursors (monomers) form; a homogeneous nucleation occurs when the concentration of these monomers reaches a certain supersaturation level (C_s). The saturation further increases to a level (C_{min}), at which activation energy barrier for nucleation can be overcome leading to rapid self-nucleation (burst nucleation).¹⁴ Due to this burst nucleation, the supersaturation levels lower immediately below this self-nucleation level ending the nucleation period; growth then occurs due to diffusion of the monomers in the solution towards particle surfaces interpreted as heterogeneous nucleation.¹³ Other than diffusion limited growth, NPs growth can also occur via aggregation or coalescence and Ostwald ripening.

The LaMer model and its modifications are still the only commonly accepted models describing the general mechanism of the NP formation process. For the size adjustment of colloidal NPs, the model rationalizes some conventional strategies such as fast nucleation of molecular precursor and slow growth rate to achieve monodispersity, increasing concentration of stabilisers to get smaller NPs etc. Because the physical properties of NPs are strongly dependent on their size, shape and size distribution, different synthetic pathways

have been reported to produce IONPs with narrow size distributions and good magnetic properties.

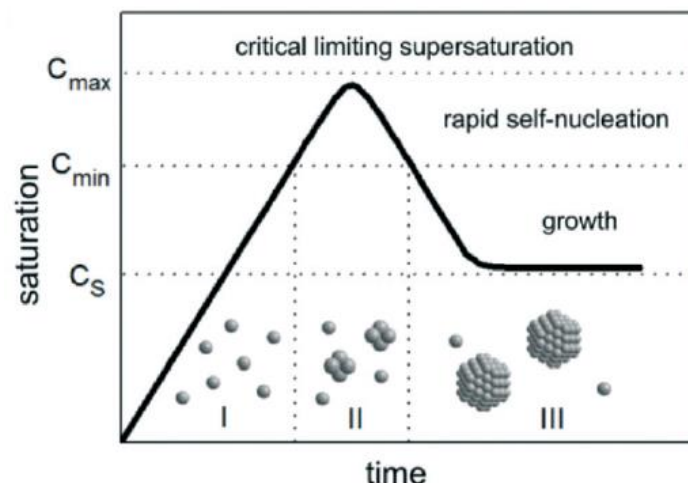


Fig. 1.1. The principle of NP nucleation: LaMer's mechanism of nucleation. The (theoretical) qualitative curve describes the monomer concentration as a function of time where C_s represents supersaturation concentration, C_{min} represents the minimum concentration limit above which a burst nucleation happens and C_{max} represents critical limiting supersaturation.¹⁵

Co-precipitation method is the most commonly employed approach to obtain water dispersible IONPs. However, NPs agglomeration and polydispersity are the main issues with co-precipitation approach. They can be addressed by hydrothermal method of co-precipitation in a pressure vessel at elevated temperatures (100°C - 250°C) but the NPs formed are reported to have irregular morphologies. The two other methods (polyol and thermal decomposition) give better monodispersity and are discussed in more detail below.

1.1.1. Thermal decomposition of organometallic precursors

Thermal decomposition of various organometallic complexes such as iron acetylacetonate ($\text{Fe}(\text{acac})_3$),¹⁶ iron oleate ($\text{Fe}(\text{oleate})_3$)¹⁷ and iron carbonyl (iron pentacarbonyl ($\text{Fe}(\text{CO})_5$))¹⁸ in apolar organic solvents in presence of ligands (oleic acid and oleyl amine) is reported as a

procedure to synthesize IONPs with well-defined shapes and sizes. These syntheses are performed at reflux temperature of high boiling point apolar solvents, commonly octyl ether (288 °C), benzyl ether (298 °C) or 1-octadecene (318 °C). The ligands in the synthesis are used as stabilisers chemisorbed on the surface of the magnetic NPs. They can favour a specific morphology of the NPs by blocking the growth of certain crystallographic facets and at the end of reaction, they assist the NPs dispersion and prevent aggregation by pointing their hydrophobic chains outside into the solvent.

In case of a zerovalent metal precursor ($\text{Fe}(\text{CO})_5$), the thermal decomposition initially leads to the formation of metal NPs, but a two-step procedure can be used to produce IONPs. This two-step procedure was originally introduced by Heyon *et. al.*¹⁸ In the first step, $\text{Fe}(\text{CO})_5$ is decomposed in a mixture of octyl ether and oleic acid at 300 °C to generate iron nanoparticles and then, a mild oxidant, trimethylamine oxide ($(\text{CH}_3)_3\text{NO}$) is added at an elevated temperature to generate $\gamma\text{-Fe}_2\text{O}_3$ (~11 nm).¹⁸ Iron NPs are prone to oxidation and oxidise in air to form iron oxides even without oxidants. Decomposition of Fe^{3+} precursors ($\text{Fe}(\text{acac})_3$, $\text{Fe}(\text{oleate})_3$) leads directly to the oxides, that is, to Fe_3O_4 , by a simple one-step procedure introduced by Sun *et. al.*¹⁶ They synthesized various sized Fe_3O_4 NPs (3 – 20 nm) from the thermal decomposition of $\text{Fe}(\text{acac})_3$ in the presence of 1,2- hexadecanediol, oleylamine, and oleic acid in phenol ether. Organic alcohols and amines act as reducing agent to reduce some of Fe^{3+} to Fe^{2+} , which then react to form magnetite. It has been hypothesized that, irrespective of the source of iron ($\text{Fe}(\text{CO})_5$, $\text{Fe}(\text{acac})_3$ etc.), above pyrolysis temperature, iron oleate acts as the real precursor.¹⁹ Iron oleate complex can be produced before reaction from the inexpensive $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ but it must be purified beforehand in order to remove chlorine anions from the medium.²⁰ Hence, $\text{Fe}(\text{acac})_3$ becomes the most prominent precursor to synthesize IONPs using this procedure.

Thermal decomposition offers an adequate control on the particle size over a wide range with good monodispersity. Moreover, by controlling the synthesis parameters, several morphologies can be obtained from perfectly spherical to slightly polyhedral²³ or prismatic²⁴ and cubic.^{25,26} The NPs obtained after the thermal decomposition are not dispersible in polar solvents, which is a limitation for biological applications. Following a ligand-exchange strategy, fatty acids can be exchanged with polar molecules, such as citric acid,²¹

tetramethylammonium hydroxide (TMAOH) etc.²⁷ Amphiphilic polymers can also be used as phase transfer agents while keeping a good size-dispersity and colloidal dispersion. However, these post synthesis steps to render the NPs hydrophilic and biocompatible, are difficult to monitor and control precisely in a quantitative manner. Recently, a new route to synthesize water dispersible IONPs have been developed through a simple polyol method which involves the thermal decomposition of iron precursors ($\text{Fe}(\text{acac})_3$, $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in a polyol solvent. More detail on polyol approach is reported in the section below.

1.1.2 Polyol method

Polyol synthesis is an alternative where synthesis of the IONPs is done in the presence of polyol solvents such as diethylene glycol (DEG), 1,2-propylene glycol (PG), or ethylene glycol (EG). Thanks to the high boiling point of polyols, NP synthesis can be carried out at high temperatures resulting in monodisperse NPs with better crystallinity. These polyol solvents stabilize metal precursors due to their chelating properties and also prevent NPs from agglomeration.

For the magnetite synthesis using $\text{Fe}(\text{acac})_3$, reduction of some Fe^{3+} is needed to produce Fe^{2+} precursors. The polyols are known to reduce some Fe^{3+} to Fe^{2+} precursors, which react to form mixed metal oxide magnetite.²⁸ Maity *et. al.* synthesized magnetite NPs by this approach under inert atmosphere.²⁹ They obtained highly crystalline NPs with a diameter of 11 nm. Hachani *et. al.* observed a correlation between the length of glycol and size of NPs, as the greater the length of the glycol, the larger the size of the synthesized NPs (**Fig. 1.2**).³⁰ These studies confirmed that the choice of the polyol solvent is critical to obtain high quality nanoparticles with a desired size and with a narrow size distribution.

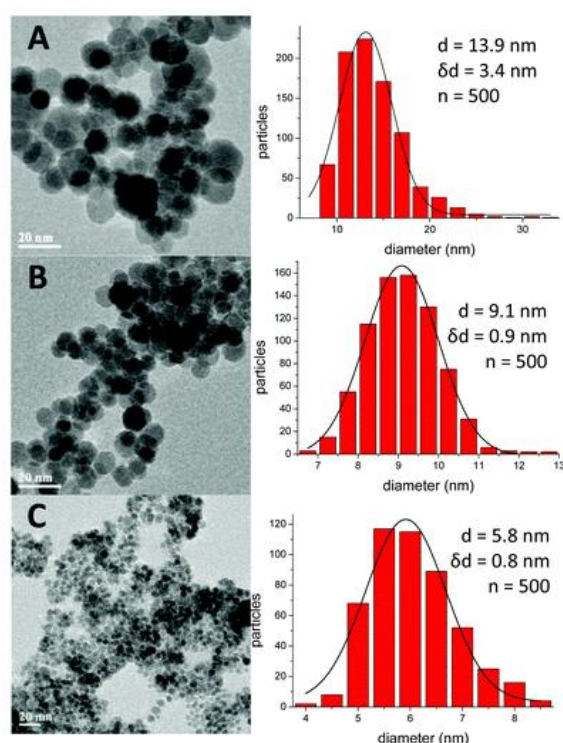


Fig. 1.2. TEM images and particle size distributions of iron oxide nanoparticles synthesized using different polyols (A) tetraethylene glycol (TEG), (B) triethylene glycol (TREG) and (C) DEG. Here, d = mean diameter, δd = standard deviation and n = number of particles counted.³⁰

In the following section, approaches to obtain stable water dispersible NPs suspensions, which is an essential requirement for biomedical applications are reported.

1.2. IONPs stabilization: Electrostatic and steric stabilization

In general, particles at the nanoscale are unstable and tend to agglomerate because of van der Waals, electrostatic or magnetic forces to minimise the overall surface free energy.³¹ Without any counteractive repulsive force, the aggregation would lead to bulk precipitates rather than NPs. Such repulsive forces can be achieved by electrostatic stabilization or steric stabilization of the NPs³² which are described in detail below.

1.2.1. Electrostatic stabilization

Citric acid is the most common stabilizer used for the electrostatic stabilization of IONPs. As ions adsorb on the NP surface, electrostatic repulsion prevents particles from coming close to each other and aggregating. In aqueous solutions, the diffuse double layer (DDL) extends far from the particle surface, facilitating particle–particle repulsions. However, at high ionic strengths, the NPs DDL is compressed and neutralized with the subsequent aggregation due to van der Waals forces. Thus, electrostatic stabilization largely fails to provide sufficient colloidal stability in biological media. The second method consists in the generation of a physical barrier at the NP surface (i.e., steric stabilization). For instance, polymers attached to the particle surface (e.g., poly(ethylene glycol), PEG or chitosan) are often used to increase NPs stability in suspensions.³³

1.2.2. Functionalization approaches for the steric stabilization of IONPs

The presence of hydroxyl groups, such as Fe-OH, on the IONPs surfaces, open various possibilities to attach different functionalities to the NPs surface by exploiting electrostatic, hydrophobic, chelating and covalent interactions (**Fig. 1.3**).³⁴ Modification of IONPs using alkoxysilane compounds is one of the most commonly used surface modification technique where silane can be covalently attached onto IONP surfaces by reaction of the surface Fe-OH group with the Si-OCH₃ moiety.^{35,36} Silane functionalization involves further cross-linking events to produce a thin inorganic silica layer around the particles but during this process, the irreversible formation of aggregates can be observed.³⁷ Another widely employed functionality for the surface modification of IONPs is carboxylic acid group (–COOH), which can interact with the surface of IONPs by coordination processes. Citric acid has been used commonly for the stabilization of IONPs.²¹ IONPs synthesized in carboxylic acids such as oleic acid can also lead to the formation of a carboxylate layer on the surface.^{21,22} However, coordination bond between –COOH and IONPs can be broken easily by increasing temperature or by exchange with another carboxylic acid compound.²¹

Phosphonic acid can also be used to functionalize IONPs surface due to the formation of more stable Fe–O–P– bonds as compared to carboxylic acid bond.^{38,39} Finally, dopamine can be used to functionalize IONPs surfaces due to the strong interaction of catechol moieties with the iron due to chelate effect.⁴⁰⁻⁴¹ This approach has been widely used to attach a range of

biologically important molecules, such as peptides and amino acids to IONPs.⁴⁰⁻⁴¹ While dopamine derivatives are the most extensively used linkers for the conjugation onto IONPs, the use of dopamine has some limitations. Fe-catechol binding is extremely strong and the catechol could etch IONPs, i.e., it desorbs taking Fe ion followed by oxidation to a quinone-like structure. Reimhult *et. al.* demonstrated that this limitation can be overcome by attaching electronegative nitro-groups to the aromatic catechol system.⁴² The introduction of nitro-groups renders the dopamine ligand electron deficient and increases the oxidation potential of the 2-nitrocatechol ligand, resulting in a high and irreversible binding affinity to IONPs. Hence, nitrodopamine (NDA) and phosphonic acid groups appear to improve IONP stability over a larger range of pH and temperature compared with carboxylic acid groups.

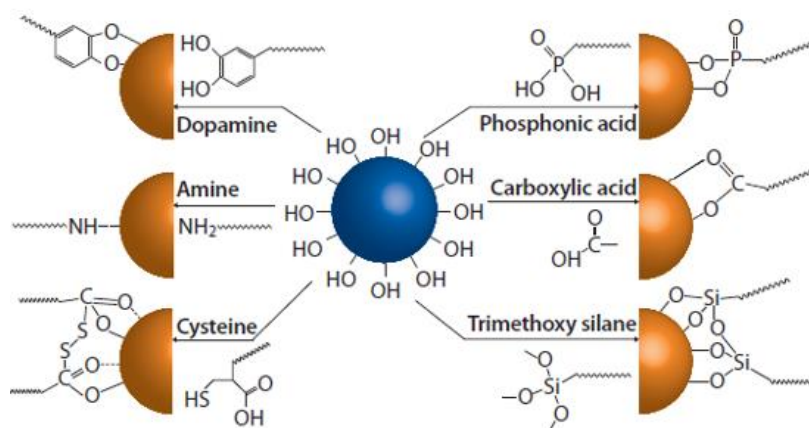


Fig. 1.3. Functional groups that can be used to anchor polymers on IONPs surface.³⁴

1.2.3. Steric stabilization with polymers

Steric stabilization of IONPs with a polymer coating is preferred for many medicinal applications. A polymer coating can provide multiple functionalities on the NPs surface allowing the scope of designing hybrid NPs having the capacity of targeting, tracking, delivery and stimulated release of therapeutic drugs (peptides, proteins etc.).⁴³⁻⁴⁵ Some of the common polymers used to stabilize IONPs are given below (**Fig. 1.4**).

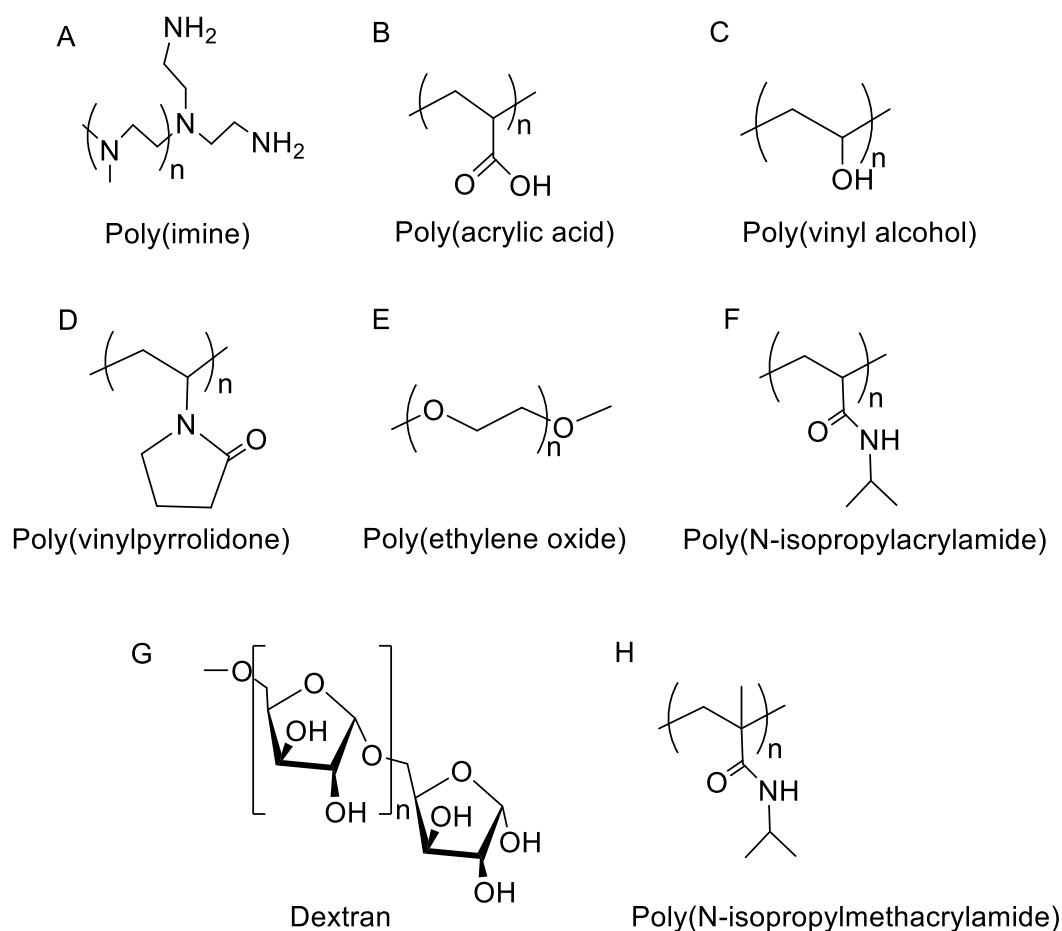


Fig. 1.4. Polymers used for IONPs stabilization. A and B are ionic polymers.

The main modes of attachment of polymers to IONPs are: a) through binding groups present in the repeat unit; b) via a polymer end group. Polymers having Fe-binding groups in their repeat units (e.g., poly(acrylic acid)) can be directly coated on to NPs surface (chelate effect).⁴⁶ The attachment of polymer chains to IONPs can also be achieved by a multiple attachment strategy using random or block copolymers.^{45,47} Due to insertion of several functional groups along the copolymer backbone, the number of possible anchoring points increase for IONPs attachment. Presence of multiple anchoring points on the backbone can also result in interaction with several particles leading to flocculation. Hence, the control of the polymer architecture is a crucial factor influencing NP aggregation.

End-group-attached polymer functionalization of IONPs can be achieved by two approaches: grafting 'to' and grafting 'from'.^{34,43} In the grafting 'from' method, polymer is directly grown

on the surface of IONPs, while in grafting 'to' approach, pre-synthesized polymer is grafted onto IONPs *in situ*.⁴³ Both approaches have their advantages and disadvantages. Higher grafting density can be achieved by grafting 'from' whereas, grafting 'to' allows better control over polymer architecture and functionality. Khurzhals *et. al.* synthesized thermally responsive poly-*N*-isopropylacrylamide (PNIPAM) coated magnetite NPs exploiting both grafting 'to' and grafting 'from' methods using atom transfer radical polymerization (ATRP).⁴³ In grafting 'to' approach, they functionalized PNIPAM polymer with NDA which acts as an anchor to attach to IONPs surfaces. Recently, our group have used this grafting 'to' approach to obtain thermally-responsive PNIPAM coated IONPs for protein delivery applications.⁴⁸ Palma *et. al.* used silane functionalized polymers to stabilize IONPs.³⁷ Gao *et. al.* used cysteine-terminated polyethylene glycol (PEG) anchored to the surface of IONPs.⁴⁴ The presence of both carboxylic acid and thiol groups allows the anchoring of the polymers and simultaneous cross-linking of these polymers through oxidation of the thiol groups to disulfide around the nanoparticles.⁴⁴

In the next section, various techniques to characterize NPs size distribution, crystallinity, and chemical composition have been discussed. Consequently, some approaches to characterize core-shell NPs are also explained. Ultimately, common techniques used to assess the magnetic properties of IONPs have been discussed. .

1.3. IONPs characterization

Iron oxide nanoparticles (IONPs) exhibit distinct electronic, optical and chemical properties as compared to their bulk counterparts. These properties are largely dependent on their size, size distribution, crystallinity and chemical composition which needs to be determined prior to any further studies.³⁶⁻⁴¹ There are various techniques to characterise IONPs; size, size distribution and morphology characterization using electron microscopy; size heterogeneity analysis using light scattering method (e.g., DLS); crystal structure, grain size and elemental composition analysis using X-ray based techniques (e.g., XRD, XPS); and, magnetic characterization using magnetometry methods (e.g., VSM). Extent of the surface coverage (grafting density) of organic layer on the NPs surface can be calculated using

thermogravimetric analysis (TGA). Some of these techniques are described in the following sections.

1.3.1. Size, size distribution and morphology analysis of IONPs

Since, NPs have a size below the diffraction limit of visible light (200-250 nm), optical microscopy can't be used for their size characterization. Electron microscopy is largely used for size analysis of NPs and numerous microscopy techniques are commercially available, however transmission electron microscopy (TEM) and scanning electron microscopy (SEM) are arguably the most popular for NPs analysis. The resolution of SEM is relatively low (10 nm), hence, it can only be used to analyse NPs with size greater than 10 nm. TEM is the most common technique to analyse NPs size and shape because of its high resolution (0.05 nm). It provides the direct images of the sample which can be analysed to study the size, size distribution and homogeneity of the NPs. This technique has some limitations; difficulty to quantify a large number of particles (high concentration); misleading images due to orientation effects; and, aggregation during the drying of the colloid suspension on TEM grids. Hence, extra care should be taken during the sample preparation, since an inadequate protocol can result on sample alteration or artefact creation. TEM is usually combined with other techniques that can measure larger numbers of particles, and require less sample preparation, such as dynamic light scattering (DLS, see details in later section).²⁹ High-resolution TEM (HRTEM) is an imaging mode of transmission electron microscopy which uses the phase-contrast imaging technique to detect the arrays of atoms in crystalline structure. HRTEM has become the most common technique to characterize the internal structure of NPs since, it provides the information about the single particle crystal structure and polycrystallinity of the NPs system.³⁶⁻⁴¹ Selected area electron diffraction (SAED) is another crystallographic experimental technique that can be performed inside TEM which gives the information about the polycrystallinity of the NPs sample.⁴⁹ In addition to size, shape and size distribution, the crystal structure of the NPs and their chemical composition need to be investigated thoroughly. Energy dispersive X-ray (EDX) microanalysis is an elemental analysis technique associated with TEM based on the generation of characteristic X-rays corresponding to specific elements present in the specimens.⁵⁰ X-ray diffraction (XRD) is a common technique used for the characterization of NPs crystalline structure and, is described below.

1.3.2. Crystal structure, grain size and elemental composition analysis

XRD provides the information about the nature of the phase, lattice parameters and crystalline grain size of the NP. Scherrer's equation (**equation 1.1**) is used to calculate the grain size of the NPs using the broadening of the most intense peak of an XRD measurement for a specific sample.⁵¹ XRD is a non-destructive bulk technique commonly performed on powdered samples, results in volume-averaged values.

$$\tau = \frac{K\lambda}{\beta \cos\theta} \quad (1.1)$$

Equation 1. Scherrer's formula to calculate grain size. Here, τ is mean size of ordered domain, K is dimensionless shape factor, λ is X-ray wavelength, θ is Bragg angle and β is full width at half maxima of peaks (β) in radian located at any 2θ in the pattern.

It is worthy to mention that, the Scherrer formula often underestimates the particle size. The reason for this is that a variety of factors besides instrumental errors and crystallite size can contribute to broadening of diffraction peaks. The common sources of peak broadening could be dislocations, twinning, chemical heterogeneities or grain boundaries etc. If all these contributions including instrumental broadening, were zero, then the Scherrer formula would apply and peak width would represent the crystallite size. The composition of the particles (crystalline phase) can be determined by comparing the position and the intensity of the peaks with the references patterns available at the International Centre for Diffraction Data (ICDD, previously known as Joint Committee on Powder Diffraction Standards, JCPDS) database. However, it is not suitable for amorphous materials and the XRD peaks are too broad for particles with size below 3 nm. Moreover, in the case of IONPs, it is not useful in differentiating between different iron oxide phases (magnetite and maghemite) due to their similar XRD patterns (**Fig. 1.5**).⁵²

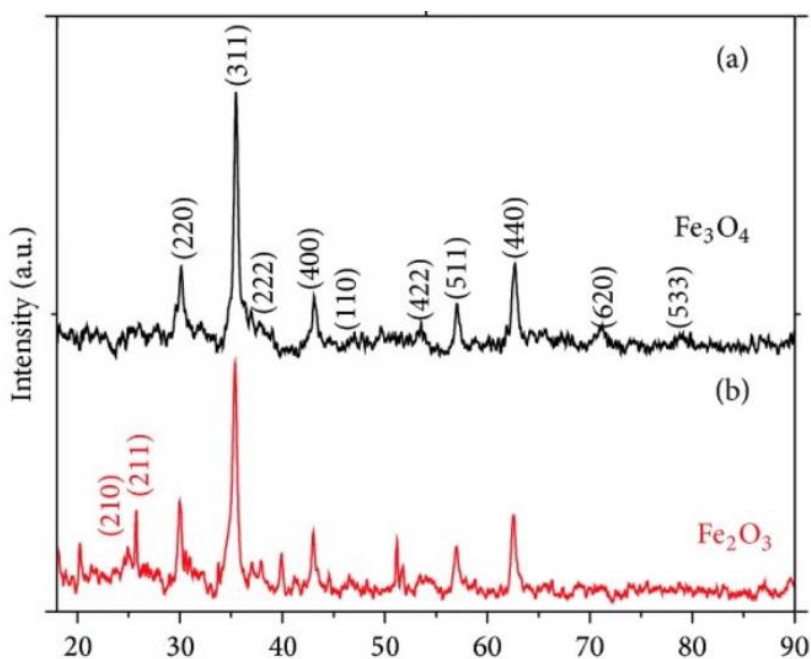


Fig. 1.5. Powdered XRD patterns for magnetite (a) and maghemite (b).⁵²

Size, size distribution, shape and crystallinity have a major impact on the NPs properties, but organic ligands present on the surface of the particles may also affect some properties and possible applications of the NPs. Some techniques that can be used to characterize core-shell NPs (ligand-coated NPs) are described below.

1.3.3. Other techniques to characterize core-shell NPs

There are several other techniques to characterize core-shell NPs. Fourier-transform infrared spectroscopy (FTIR) is commonly used to characterize the ligands present on the NPs surface. It is a quick technique to monitor ligand exchange in the case of NPs. Mohapatra *et al.* used FTIR to monitor the ligand exchange from oleic acid to citric acid to get water dispersible magnetite NPs (**Fig. 1.6**).²¹ In comparison with the as-synthesized oleic acid/oleyl amine coated IONPs, the citric acid coated Fe_3O_4 samples show strong IR peaks of the symmetric stretching of COO^- (1390 cm^{-1}), symmetric stretching of C-O (1606 cm^{-1}), and C-OH stretching (1046 cm^{-1}) groups of citric acid which confirmed the citric acid functionalization on the surface of Fe_3O_4 nanoparticles (**Fig. 1.6**).

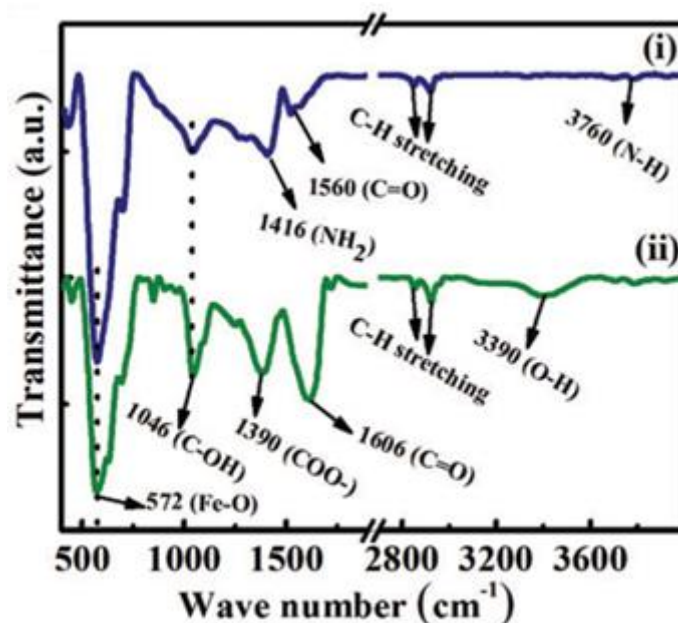


Fig. 1.6. Comparative analysis of the FTIR spectra of: (i) as prepared magnetite and (ii) citric acid modified magnetite nanoparticles of average size 28 nm.²¹

FTIR offers information about the confirmation of the stabilizer type on NPs surface but it does not give insight about the extent of surface coverage or mass ratio of NP to stabilizer. In case of IONPs, this ratio is needed to normalize the values of saturation magnetization to purely metallic content. Thermal gravimetric analysis (TGA) can be used to determine the mass and composition of the stabilisers present in a nanomaterial sample. In this technique, a sample is heated slowly and components with different degradation temperature decompose and vaporise, and a change of mass is recorded. The temperature and corresponding mass loss recorded by TGA can be used to quantify the amount of organic ligand present in the known mass of the starting sample.⁴³

Nuclear magnetic resonance (NMR) spectroscopy can also be used to study the interactions or coordination between ligand and surface of diamagnetic or antiferromagnetic NPs. NMR is not useful to characterize ferri- or ferromagnetic materials (e.g., magnetite, maghemite, ferrites etc.) due to their large saturation magnetization, which causes variations in local magnetic field and hence significant signal broadening.⁵¹ For core-shell NPs, one way to characterize the shell would be to dissolve the NPs (remove all Fe^{3+}), and then use NMR to characterize the organic material (polymer).

NPs in suspension behave differently (may associate or dissociate) to the powdered form and can be monitored by dynamic light scattering (DLS). It is a widely used technique to monitor the particle size in colloidal suspensions in the nano- and sub-micrometer ranges.²⁹ DLS gives the information about the hydrodynamic diameter (diameter of the NP and the solvent molecules that diffuse at same rate) and a size distribution profile of the NPs in suspension can be obtained through Stokes-Einstein assumptions.⁵¹

In the next section, the use of IONPs in MH is briefly discussed along with various safety parameters for the *in vivo* applications. Additionally, different heating mechanisms and important parameters that affect magnetic heating efficiency of IONPs have also been discussed.

1.4. Magnetic hyperthermia

The term hyperthermia in medical oncology, refers to a therapeutic method by which a given area of interest is subjected to a temperature (T) higher than 40 °C.^{53,54} The history of oncological hyperthermia started from some evidence of cancer cure by concomitant febrile diseases described in XVIII-XIX centuries.⁵⁵ A more recent modality is the MH, where the temperature surge is achieved by applying an AMF to a magnetic material placed in the body, mostly iron oxide.^{21-26,,29,56-61} The use of IONPs as a minimally invasive agent in MH was first addressed by Gilchrist *et. al.* in 1957.⁶² For *in vivo* application of MH, this seminal work pointed out some crucial challenges which are still under discussion in the scientific community: (i) the optimal particle dose to achieve maximum heat release; (ii) safety limit of the AMF; (iii) reliability for providing a precisely controlled tumour specific heat exposure mediated by IONPs.⁶³

1.4.1. Safety limits of AMF for *in vivo* applications

Various groups have investigated the important operational parameters to effectively carry out magnetic hyperthermia in cancer therapy.⁶⁴⁻⁶⁷ Two different safety limits have been established to limit the amplitude and frequency of the AMF in order to reduce the effects of

non-specific eddy currents. Eddy currents are electrical currents that are induced within the conductor, in this case the human body, due to the changing magnetic field, as described by Faraday's law of induction. Excessive non-specific heating of normal tissues by eddy currents is the primary determinant of the maximum tolerable field strength and frequency.⁶⁵ First limit was established in 1984 by Atkinson *et. al.*,⁶⁶ according to which the product of the AMF amplitude and the frequency must be less than or equal to 608.2 Ts^{-1} . In chemical context, the MRI scanners operates at 501600 Ts^{-1} (strength = 0.5 T, frequency = 1 MHz), which is much higher than this tolerance limit. Therefore, Hergt and Dutz in 2006 increased this tolerance limit to 6270 Ts^{-1} .⁶⁷

The first clinical magnetic hyperthermia treatment system was developed at Charité – Medical University of Berlin in 2004 for the treatment of prostate cancer (2257.2 Ts^{-1}).^{68,69} In the first patient treated, maximum and minimum intra-prostatic temperatures measured at a field strength of $4.0\text{--}5.0 \text{ kA m}^{-1}$ ($0.005 \text{ T} - 0.006 \text{ T}$) were $48.5 \text{ }^{\circ}\text{C}$ and $40.0 \text{ }^{\circ}\text{C}$ ($\Delta T \sim 9 \text{ }^{\circ}\text{C}$) during the 1st treatment and $42.5 \text{ }^{\circ}\text{C}$ and $39.4 \text{ }^{\circ}\text{C}$ ($\Delta T \sim 3 \text{ }^{\circ}\text{C}$) during the 6th treatment, respectively. Magnetic heating instrument used in our study (2884.2 Ts^{-1}) is designed within this tolerance limit and hence $\Delta T \geq 9 \text{ }^{\circ}\text{C}$ was expected.

1.4.2. Heating mechanisms in superparamagnetic IONPs

In an AMF, IONPs generate thermal energy due to the change in the magnetic moment from the preferred orientation and the subsequent relaxation to equilibrium. Heating in magnetic nanoparticles (MNPs) can occur mainly via two mechanisms: (1) hysteresis loss and (2) relaxation losses.⁷⁰ Hysteresis losses occur in bigger IONPs which contains multiple magnetic domains. When such particles are subjected to an AMF, the orientation of the magnetic moments align continuously with the direction of the magnetic field (**Fig. 1.7**). This results in a difference in energy that is released in the form of heat. As NPs size decreases, the number of magnetic domains also decrease until a single magnetic domain remains at a threshold size ($\sim 20 \text{ nm}$).^{21,22} Below this size, MNPs are considered superparamagnetic and in the presence of an AMF, heat is mainly produced by Néel relaxation and Brownian relaxation. Néel relaxation refers to the relaxation of the particle magnetic moment to its equilibrium orientation (**Fig. 1.7**). The rapid realignment is opposed by the electronic properties of the

atoms (the spin of the Fe), resulting in heat generation. Brownian relaxation refers to the frictional heat generated from the physical rotation of particles within a supporting medium when the particles attempt to realign themselves with the changing magnetic field. A more comprehensive discussion on the mechanism of heating is beyond the scope of this chapter and covered elsewhere.⁷⁰

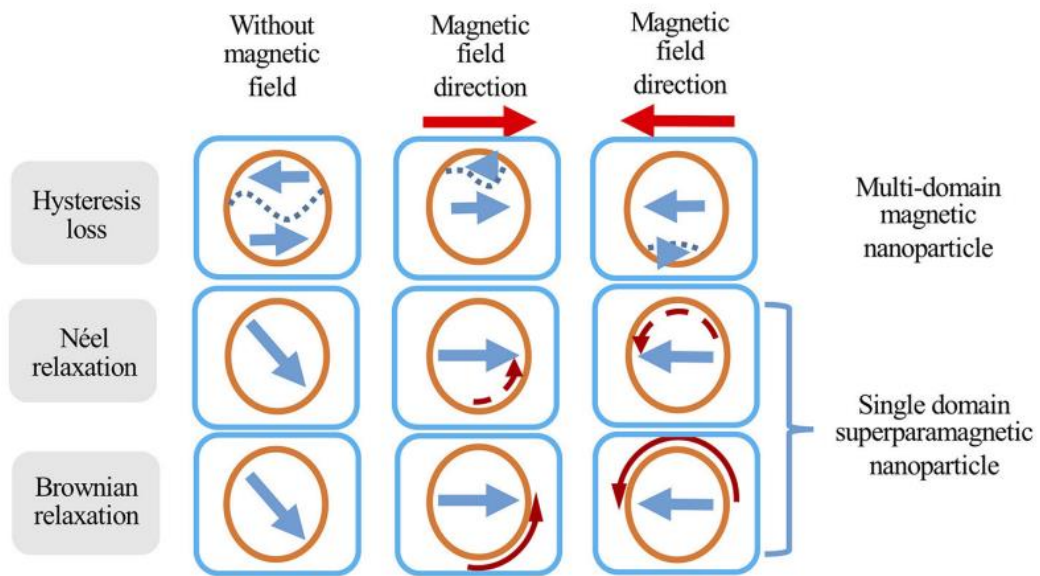


Fig. 1.7. Different heat generation mechanisms of MNPs in response to an AMF. Orange circles represent MNPs, short straight arrows represent magnetic field direction, curved arrows represent the movement (solid curved arrow) or change in magnetic moment direction (dashed curved arrow), and dashed lines represent domain boundaries in multi-domain particles.⁷⁰

1.4.3. Heating efficiency measurement of MNPs in AMF: SAR determination

The heating efficiency of the MNPs is represented by SAR (also referred as specific loss power (SLP)),⁷¹ which is defined as the ratio of the power dissipated and the mass of the NPs (W/g). The equation for calculating the SAR value is shown below (**equation 1.2**).

$$SAR = \frac{1}{m_{Fe}} C_{sol} m_{sol} \frac{dT}{dt} \quad (1.2)$$

Where m_{Fe} is the mass of iron in the sample (g), C_{sol} is the specific heat capacity of the solvent ($C_{\text{H}_2\text{O}} = 4.184 \text{ J } ^\circ\text{C}^{-1} \text{ g}^{-1}$), m_{sol} is the mass of solvent (g) and $dT(^{\circ}\text{C}) / dt \text{ (s)}$ is the temperature gradient vs time.

One limitation of the SAR representation is its dependence on the amplitude and frequency of the AMF, which makes direct comparison of reported literature values difficult owing to variations in the applied AC field conditions. The characteristic SAR value for MNPs is determined by multiple variables such as the magnetic properties of the MNP, structure and size of the magnetic core, surfactants, dispersion medium, monodispersity, inter-particle interactions and the frequency and amplitude of the AMF. Some of these factors are discussed in detail in the next section.

1.4.4. Factors affecting heating efficiency of IONPs

1.4.4.1. Effect of size of IONPs, AMF frequency and amplitude on magnetic hyperthermia properties

The power dissipated during MH measurements is dependent on the intrinsic magnetic properties of the IONPs, which influences its relaxation (Néel and Brownian) and hysteretic losses.^{72,73} The Linear Response Theory (LRT) is a widely accepted framework to analyse the power absorption of magnetic NPs for MH.⁷⁴ According to the LRT model, the heating efficiency of MNPs depends on the saturation magnetization of the MNPs, NP size (volume) and magnetic anisotropy (Neel relaxation). The saturation magnetization of IONPs decreases with decreased particle size.⁷⁵ In small IONPs, saturation magnetization decreases due to surface and internal spin canting effects.⁷⁵ These interactions give rise to magnetically disordered spin glass-like layers on the surface of magnetic NPs, hence decrease in the magnetization.⁷² The presence of a magnetically distorted layer on the NP surface formed due to incomplete coordination of the metal ions, also lowers their saturation magnetization.⁷⁶ Vreeland *et. al.* conducted MH studies on IONPs of various sizes and found that for an AMF strength (H) = 36.5 kA/m (0.05 T) and frequency (f) = 341 kHz, the optimum size showing maximum SAR was around 22 nm.⁷⁷ Mohapatra *et. al.* conducted similar studies on various NPs sizes (3-40 nm) for H = 49.7 kA/m (0.06 T) and f = 265 kHz. They observed that SAR

increases with the increase in particle size and attains a maximum at a size of 28 nm (**Fig. 1.8 (a)**), then the value decreases with a further increase in the particle size.²¹ In contrast to the superparamagnetic regime (3–16 nm), they found that the SAR values of IONPs were enhanced strongly with the increase in AMF amplitude. For example, in 16 nm sized MNPs, they observed an increase in the SAR value from 105 to 298 W/g (increased by 194 W/g) with the increase of AMF amplitude from 184 to 625 Oe (~ 0.02 T to 0.06 T) and in 28 nm sized MNPs, the SAR value increased by 504 W/g (from 297 to 801 W/g). The enhancement in the SAR value at high AMF amplitude can be attributed to the collective contribution of relaxation loss (Néel and Brownian) and hysteresis loss from the NPs (**Fig. 1.8 (b)**).²¹⁻²²

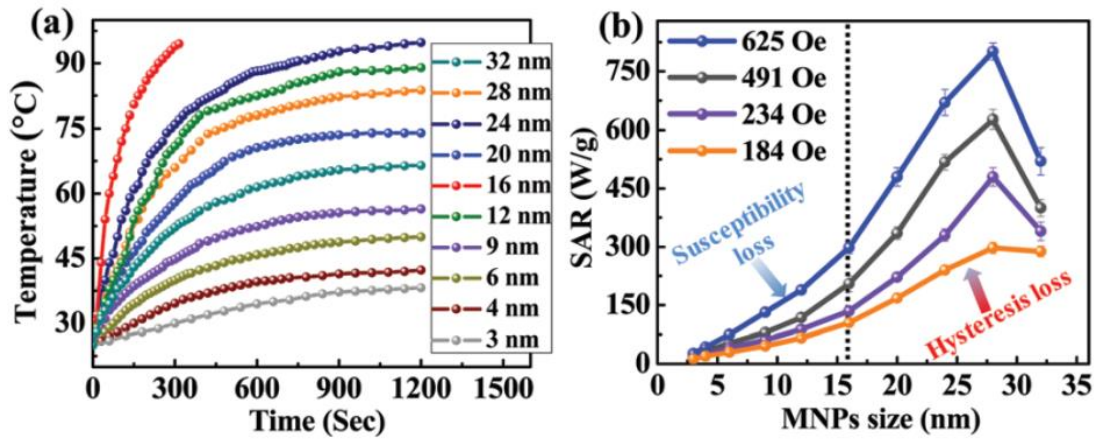


Fig. 1.8. (a) Temperature variation as a function of time for aqueous suspension (2 mg/ml) of Fe_3O_4 MNPs (3–32 nm) under the AMF (49.7 kA/m (0.06 T)) and (b) variation of the SAR values as a function of MNP size for different AMF fields (184 Oe = 0.018 T, 234 Oe = 0.023 T, 491 Oe = 0.049 T and 625 Oe = 0.062 T).²¹

Optimal NP size and magnetic anisotropy are influenced by the AMF strength (H) and frequency (f). Maximum absorption of magnetic energy occurs when the effective relaxation time (T) is close to the period of the excitation field.⁷⁸ This means that for a given f there is an optimum size that resonates well with the applied field.⁷⁹ Corato *et. al.* studied the effect of the AMF frequency on SAR measurement of iron oxide nanocubes (size ~ 18 nm).⁸⁰ Four frequencies were chosen for the study: 320, 500, 700 and 900 kHz at an amplitude of 24 kA/m. The SAR values were found to increase (more than 100 W/g) with each frequency increment (**Fig. 1.9**).

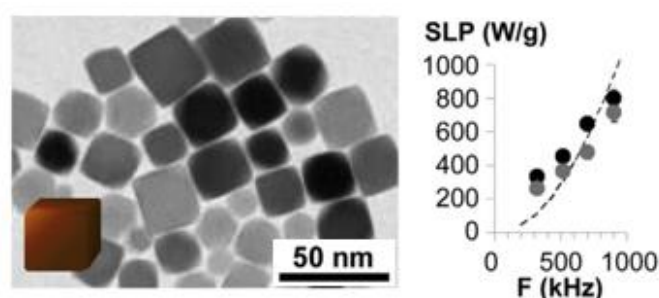


Fig. 1.9. TEM images (left) and heating capacities (right, SAR (W/g) as a function of the frequency of the applied magnetic field at an amplitude of 24 kA/m (0.03 T)) for iron oxide nanocubes (~18 nm on edge). The SAR were measured for the nanomaterials resuspended in water (black plain circles), or in glycerol (grey plain circles).⁸⁰

Table 1 represents SAR values reported in literature for various NPs and at different AMF strength and frequency.

Table 1.1. Comparison of SAR values of various IONPs under different applied magnetic field

S.No.	IONP diameter	Coating	AMF strength and frequency	SAR (W/g)
1	16 nm NPs ²¹	Citric acid	0.018 T, 265 kHz	105
2	16 nm NPs ²¹	Citric acid	0.060 T, 265 kHz	298
3	11 nm NPs ²²	Polyethylene glycol	0.012 T, 325 kHz	14.9
4	11 nm NPs ²²	Polyethylene glycol	0.026 T, 325 kHz	50.5
5	12 nm NPs ⁵⁶	Polyethylene glycol	0.047 T, 500 kHz	349
6	10 nm NPs ⁵⁷	No surface modification	0.019 T, 300 kHz	168
7	19 nm Nanocubes ²⁵	poly(maleic anhydride-alt-1-octadecene)	0.028 T, 325 kHz	1000
8	22 nm NPs ⁵⁸	poly(acrylic acid)	0.019 T, 500 kHz	716

9	14 nm NPs ⁵⁹	Pluronic F127	0.031 T, 400 kHz	447
10	35 nm Nanocubes ²⁵	poly(maleic anhydride-alt-1-octadecene)	0.030 T, 320 kHz	1391
11	8 nm NPs ⁶⁰	No surface modification	0.012 T, 100 kHz	17.2
12	24 nm NPs ⁶⁰	No surface modification	0.012 T, 100 kHz	3.7
13	8 nm NPs ⁶⁰	No surface modification	0.030 T, 100 kHz	52.8
14	24 nm NPs ⁶⁰	No surface modification	0.030 T, 100 kHz	134.1
15	14 nm NPs ³⁹	phosphate capped	0.001 T, 126 kHz	11.1
16	11 nm NPs ²⁹	triethylene glycol	20 MHz	885

AMF frequency and amplitude significantly affect SAR values of the MNPs (**Table 1.1**). In the entries 11-14, with increase in AMF amplitude, there was a large increase in SAR values of MNPs with a greater diameter (24 nm) as compared to the smaller NPs (8 nm). It can be attributed to the enhanced hysteresis losses from bigger NPs along with the susceptibility loss.⁶¹ In entry 16, a much higher value of frequency was used, results in the higher SAR value of NP than entry 4. However, due to different AMF frequency and amplitude conditions, a direct comparison between the various literature studies on different MNPs is difficult.

1.4.4.2. Magnetic anisotropy effects on MH properties

The heating efficiency of a magnetic NP is strongly dependent on their magnetic anisotropy. One approach to tune the MH properties is to optimize the magnetic anisotropy by developing NPs with different morphologies. Cubic shaped IONPs have become a desirable candidate because of their lower magnetic anisotropy compared to their spherical counterparts. Some theoretical studies demonstrated that cubic NPs have lower magnetic anisotropy compared

to spheres due to smaller amount of disordered spins as a result of the flat surface of the cube.²⁹ Bauer *et. al.* compared the heating behaviour of cubic and spherical IONPs, with similar magnetic volumes, and showed about two-fold increase in SAR for the cubic analogues (356.2 vs. 189.6 W/g).⁸¹ Khurshid *et. al.* investigated the heating efficiency of spherical and cubic exchange-coupled FeO/Fe₃O₄ nanoparticles (size ~ 20 nm).⁸² SAR of cubes was found to be higher than spheres (200 vs. 135 W/g at 47.7 kA/m and 310 kHz) and the heating efficiency increased with increase in the AMF amplitude (**Fig. 1.10** (a, b)). Saturation magnetization (M_s) of cubes was found to be 1.5 times less than that of spheres. Hence, they suggested that M_s is not the sole factor in determining the SAR and the heating efficiency of MNPs can be improved by tuning their magnetic anisotropy.⁸²

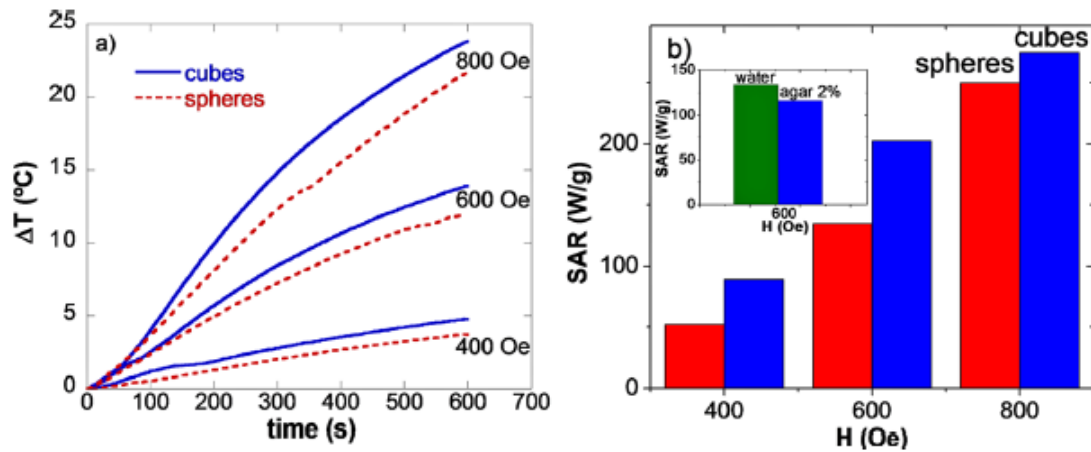


Fig. 1.10. (a) Heating curves for the spheres and cubes measured at 310 kHz and amplitudes of 400–800 Oe (400 Oe = 31.8 kA/m, 600 Oe = 47.7 kA/m and 800 Oe = 63.7 kA/m). (b) SAR values obtained from the heating curves. In the inset, the SAR values (600 Oe = 47.7 kA/m) for the nanospheres in water and in agar are compared.⁸²

An important consequence of these results is that they provide a different approach to enhance the SAR by way of modulating the magnetic anisotropy and not simply increasing the saturation magnetization of a magnetic NP.^{81,82}

1.4.4.3. Magnetic dipolar interactions: effect of aggregation on MH properties

Another important factor that needs to be considered is concentration of the IONPs. At higher concentration, due to dipolar interaction MNPs can aggregate and form nano-assemblies, which can modify their heating performance as compared to well-dispersed MNPs.⁸³ Magnetic dipolar interactions between neighbouring NPs can be regulated by introducing capping ligands that increase inter-particle spacing. IONPs with different capping agents on their surface show different heating behaviour and different SAR values.^{21-26,29,56-61} Redondo *et. al.* studied the dependence of SAR on the concentration of magnetite NPs (Fe_3O_4 NPs) along with polyacrylic acid coated magnetite NPs ($\text{Fe}_3\text{O}_4 @ \text{PAA}$).⁸⁴ For bare Fe_3O_4 NPs, they observed an increase in the SAR with increase in NPs concentration whereas in case of $\text{Fe}_3\text{O}_4 @ \text{PAA}$ NPs, SAR decreased with increase in the concentration of NPs (**Fig. 1.11**). From obtained results, they deduced that the PAA coating not only stabilized the NPs in the aqueous medium mediating the inter-particle dipolar interaction, but also changed the hydrodynamic radius of the particles and modified both Néel relaxation and Brownian relaxation processes. In case of bare magnetite nanoparticles, even at low concentration the interparticle interactions will be significant. Hence, SAR values increased with increase in NPs concentration due to significant increase in interparticle interactions with more aggregation and cluster formation at higher concentrations.⁸⁴

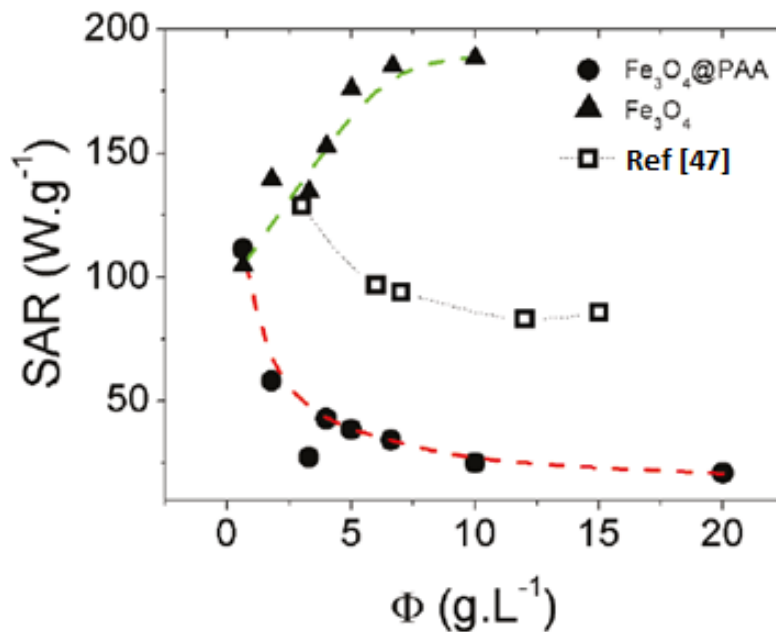


Fig. 1.11. Evolution of the specific absorption rate (SAR) of aqueous $\text{Fe}_3\text{O}_4@\text{PAA}$ NPs dispersions at several weight concentrations (Φ) between 0.6 and 20 g/L under an applied AMF amplitude of 12 kA/m and frequency of 308 kHz.⁸⁴

Levy *et. al.* observed that the uptake of magnetic NPs in sub-cellular vesicles such as lysosomes can lead to formation of magnetic NP aggregates that modify their magnetic hyperthermia properties due to dipolar interaction.⁸⁵ Mazuel *et. al.* observed a significant change in the magnetization curves of iron oxide nanocubes before and after cellular intake.⁸⁶ After cellular intake, at day 0, the hysteresis cycle opens (possibly due to agglomeration), and the magnetic susceptibility decreased, due to increase in the interparticle interactions inside endosomes (**Fig. 1.12 (a,b)**). At day 27, they observed the same tendency, albeit with slightly larger opening of the hysteresis cycle, and magnetic susceptibility was also slightly lower than at day 0 (**Fig. 1.12 (b)**). This was linked to the NP agglomeration.⁸⁶ Hence these studies are important to understand the behaviour of IONPs after cellular intake and their MH behaviour *in vivo*. In conclusion, MH is a good alternative therapeutic approach for treating cancer but in order to move MH treatment modality for *in vivo* applications, a better understanding of the behaviour of MNPs during hyperthermia measurements is required.

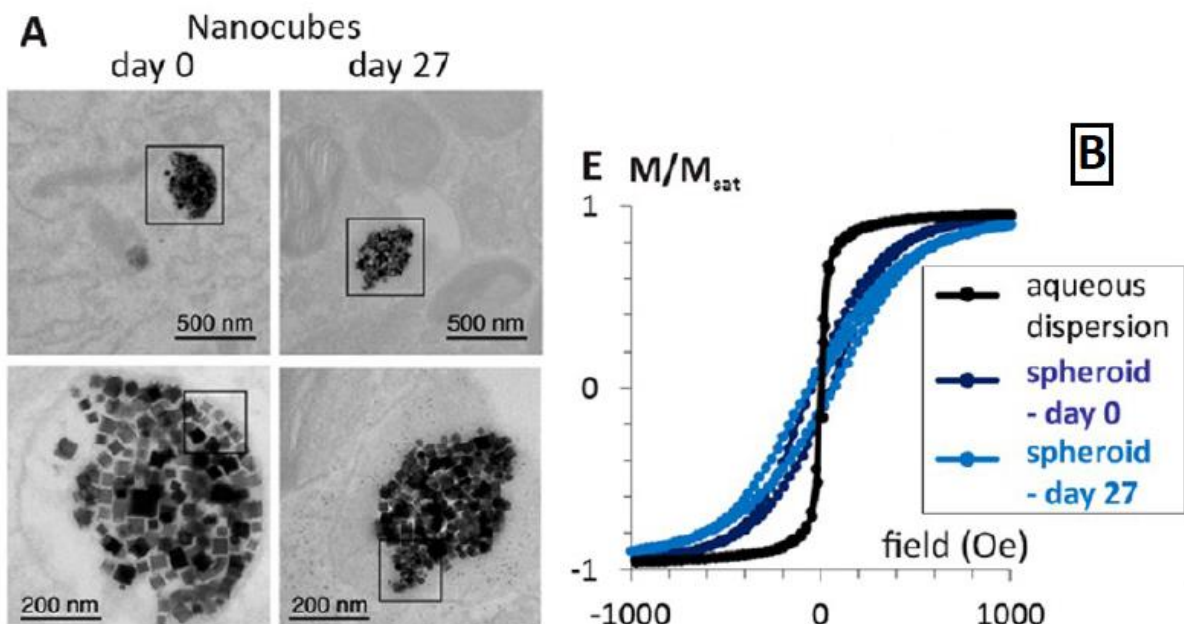


Fig. 1.12. Degradation of nanocubes **(A)** Typical TEM images of nanocubes at the time of spheroid formation (day 0) and after 27 days of maturation (day 27). **(B)** Comparison of the normalized magnetization curves in aqueous dispersion and in spheroids at day 0 and day 27 for nanocubes.⁸⁶

One of the largest applications of the NPs in nanomedicine is for the delivery of molecules to specific cells and tissues. Stimuli-responsive IONPs assemblies have attracted significant attention, due to their ability to undergo morphological or functional changes in response to stimuli.

In the next section, use of MH modality of MNPs in the magnetically triggered release of biomolecules from stimuli-responsive IONPs has been discussed.

1.5. Using MH and IONPs with thermo-responsive coatings in medicinal application

Heat is particularly attractive trigger as it can be easily applied either directly with good spatiotemporal control, or indirectly through photothermal or magneto-thermal effects. Due to their non-invasive nature and deep tissue penetration, many remote-triggering systems (NIR light, electric/magnetic fields, and ultrasound) have been proposed to induce a treatment (i.e. thermal release). Many examples of thermo-responsive polymer assemblies are reported for the trigger drug release.⁸⁸⁻⁹⁰ Aqueous solution of thermo-responsive polymer undergoes fast and reversible structural changes from a swollen to a collapsed state resulting in homogenous solution below the LCST and a phase separation above the LCST, which causes the on-off dissociation of drug molecules as a function of temperature. This thermo-responsive behaviour of the polymers can be combined with magnetic NPs, which are able to generate heat in the presence of alternating magnetic field, this could be used to stimulate the temperature-sensitive polymer to release the drug locally at the targeted sites.

Poly(*N*-isopropylacrylamide) (PNIPAM) coated IONPs has been extensively studied for their potential in controlled drug release.⁸⁸ However, the LCST of the PNIPAM polymer is below the physiological temperature (~ 32°C) which limits the *in vivo* drug delivery application. Many strategies have been developed to tune PNIPAM LCST to achieve the triggered drug release

above the physiological temperature. Dani *et. al.* developed a novel magnetic drug-targeting carrier that consisted of encapsulated magnetic IONPs with thermosensitive poly(*N*-isopropylacrylamide-co-acrylamide)-block-polyethylene amine (PNAP) coating.⁸⁹ Apart from small leak (37 °C), DOX release was high and rapid (39 °C) for the initial 5 h, followed by a sustained release for longer duration (**Fig. 1.13 (a, b)**).

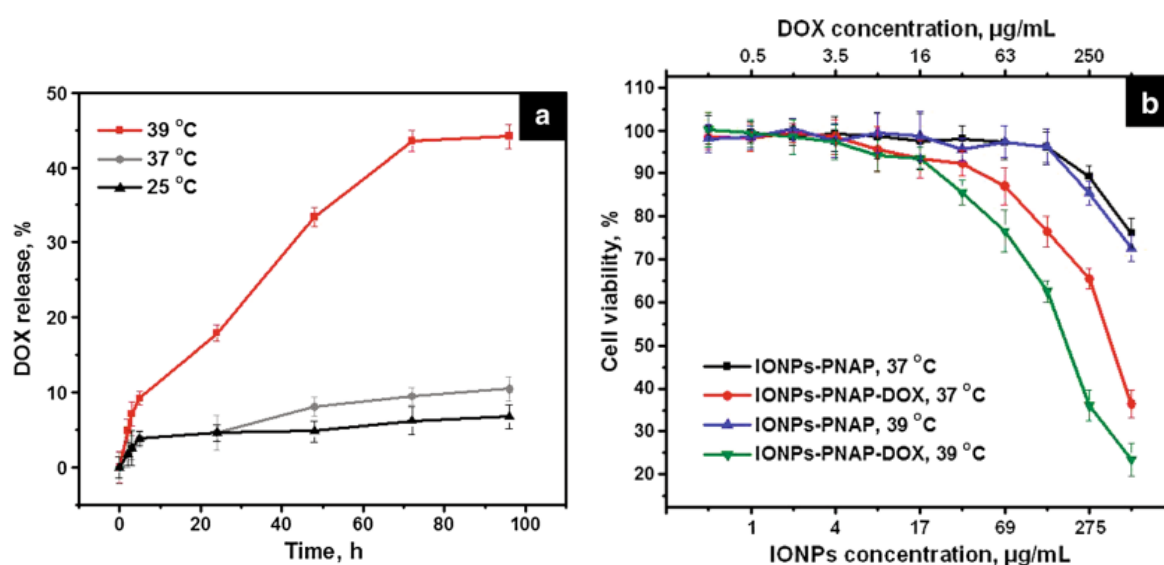


Fig. 1.13. a) The release profiles of DOX from IONPs @ PNAP incubated at 25°C, 37°C, and 39°C (temperature just above the LCST of the copolymer) in PBS buffer at pH 7.4. b) In vitro cytotoxicity at 37°C and 39°C of IONPs-PNAP (black and blue lines) and the DOX-loaded IONPs-PNAP system (IONPs-PNAP-DOX, red and green lines) against A2780/AD human ovarian cancer cells after 24 h of incubation.⁸⁹

Hoare *et. al.* developed nanocomposite membranes based on PNIPAM-based nanogels (LCST ~ 40 °C) and magnetite NPs to achieve “on-demand” drug delivery (sodium fluorescein) upon the application of an oscillating magnetic field (**Fig. 1.14**).⁹⁰ A ~20-fold higher flux of sodium fluorescein occurred at temperatures exceeding the LCST of the nanogels. The fluorescein flux could be switched on and off over multiple thermal cycles with high reproducibility, suggesting that the nanogel phase transition inside the membrane pores was fully reversible.

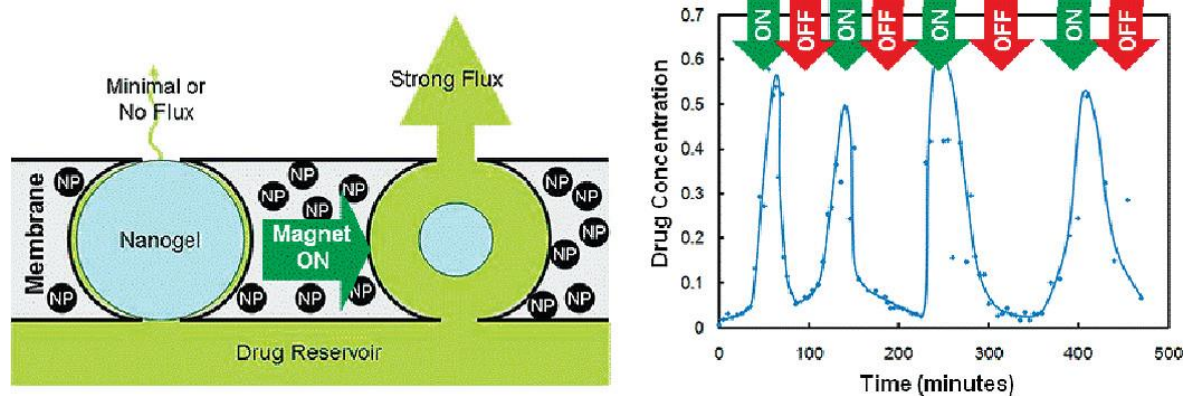


Fig. 1.14. Scheme of the proposed mechanism of membrane function, and a plot of sodium fluorescein concentration versus time over four successive on/off cycles of the external magnetic field.⁹⁰

These studies showcase the potential of thermo-responsive IONPs for the controlled drug delivery applications. However, focus has largely been directed towards the delivery and release of simple drugs or short peptides over more complex proteins.^{4,48} It could perhaps be due to the inherent functional sensitivity of proteins to factors such as temperature and pH, which are common triggers to release biomolecules from IONPs coated with thermo-responsive polymers. Recently, our group reported a successful protein encapsulation and magneto-thermal release from PNIPAM @ IONPs where, the triggered protein release occurred following local heating of the NP shell, while the bulk solution temperature remained below the LCST ($\sim 32\text{ }^{\circ}\text{C}$).⁴⁸ Conventional heating was used to check the triggered protein release above the LCST (**Fig. 1.15 (a, b)**). Additionally, the NP system was screened for the delivery of entrapped growth factor Wnt3a to the mesenchymal stem cells (MSCs). Successful Wnt3a released from coated IONPs resulted in significant increase in proliferation as compared to untreated controls after 5 days.

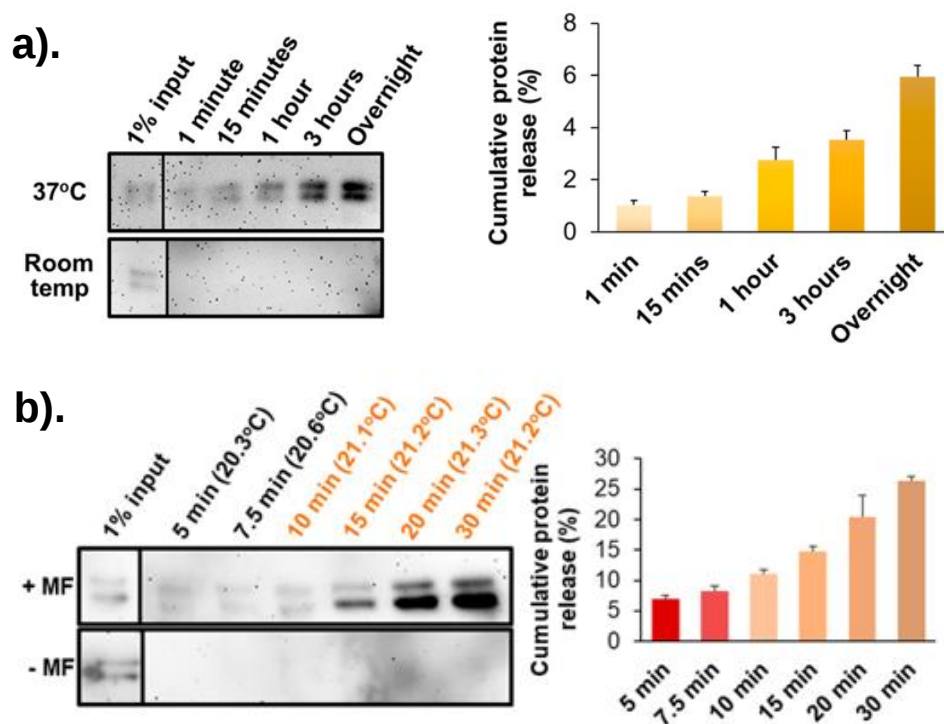
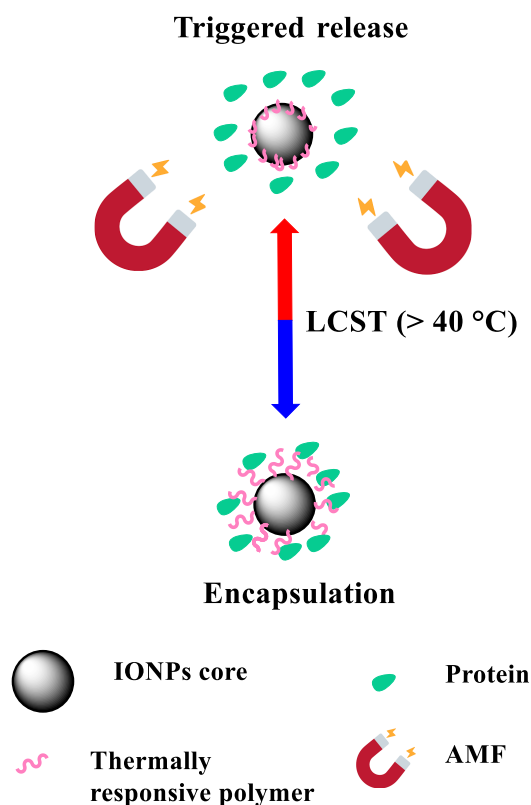


Fig. 1.15. Protein release from PNIPAM-coated IONPs: Western blot analysis and cumulative protein (apotransferrin) release (%) by **a)** conventional heating and **b)** magnetic heating of NPs. Here, MF = magnetic field.⁴⁸

1.6. Project aims

A new approach to deliver proteins for *in vivo* applications is proposed.⁴⁸ As the LCST ($\sim 32^\circ\text{C}$) of the nanocomposite (10 kDa PNIPAM @ 6 nm IONPs) wasn't appropriate for the *in vivo* applications, the main objective set for this project was to tune the LCST of the nanocomposite above 40°C (**Scheme 1.1**). This was achieved by synthesizing different molecular weight acrylamide polymers followed by their functionalization with IONPs. Earlier, the NPs obtained (6 nm IONPs) were polydisperse and had lower SAR value. Therefore, NPs with different morphology were synthesized and assessed for their heating behaviour after polymer coating. After obtaining the desired core-shell NPs (LCST $> 40^\circ\text{C}$), next objective was to check the effect of the NP properties (NP size and M_w of the polymer) on protein encapsulation/triggered release which was achieved by screening various core-shell NPs for apotransferrin (TRF) encapsulation/triggered release in the presence of a competitor protein RNaseB. After

obtaining an optimized core-shell nanostructure, effect of protein size, charge and glycosylation on their encapsulation/triggered release was studied. The main objective of these experiments was to unravel the main NP-protein interactions responsible for their encapsulation/triggered release.



Scheme 1.1. Schematic illustration of the protein delivery above 40 °C from the thermally-responsive core-shell IONPs using magnetic heating as a trigger.

In order to obtain thermo-responsive polymers with a tuned LCST, synthesis and characterization of different molecular weight polyacrylamide-based polymer has been discussed in the next chapter (chapter 2). Subsequently, synthesis and characterization of different size and shape polymer coated IONPs has been discussed (chapter 3). Chapter 4 and 5 are dedicated for studying protein-NP interactions (responsible for the protein encapsulation/release) which is an important criterion in designing any drug carrying cargo. Overall conclusion and future work are discussed in the chapter 6. Chapter 7 is on main materials and methods used in this work.

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Chapter 2: Synthesis of thermo-responsive PNIPMAM polymers

2.1. Introduction

Polyacrylamides undergo phase transition upon heating from a hydrophilic hydrated to a hydrophobic phase-separated state, at the lower critical solution temperature (LCST). This phase transition behaviour makes polyacrylamides useful in a variety of biomedical applications, such as delivery of bioactive compounds and tissue engineering.¹⁻⁵ The most widely studied polyacrylamide for such applications is poly-*N*-isopropylacrylamide (PNIPAM), with a LCST of $\sim 32\text{ }^{\circ}\text{C}$ (**Fig. 2.1**). PNIPAM stays expanded (soluble) at room temperature because of favourable (enthalpic) interactions between water and the amide groups. As the temperature increases, so does the entropic contributions to the free energy of the system, and above LCST, the polymer collapses and becomes insoluble (**Fig. 2.1**).⁶ Other polymers with thermoresponsive properties include poly(*N*-vinylcaprolactam)⁷ (LCST = $25\text{--}35\text{ }^{\circ}\text{C}$), poly(*N,N*-diethylacrylamide) (LCST = $25\text{--}32\text{ }^{\circ}\text{C}$),⁸ poly(ethylene glycol) (LCST = $85\text{ }^{\circ}\text{C}$)⁹⁻¹⁰ and poly[2-(dimethylamino)ethylmethacrylate] (LCST = $50\text{ }^{\circ}\text{C}$).¹¹⁻¹²

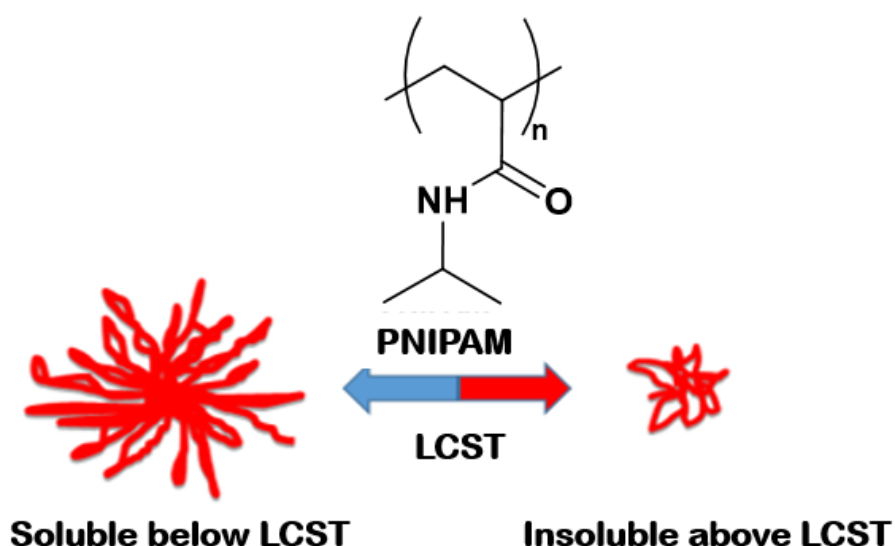


Fig. 2.1. Response of aqueous PNIPAM to change in temperature.

The modifications to LCST of these thermo-responsive polymers are important for biomedical applications because a tuned LCST would be advantageous in the controlled drug release above body temperature. The LCST of PNIPAM can be tuned between 32 °C to 50 °C by altering the monomer structure or by copolymerizing with hydrophilic or hydrophobic monomers rendering the overall hydrophilicity of the polymer higher or lower, respectively.¹³⁻¹⁴ Studies have also shown that the use of surfactants such as SDS (sodium dodecyl sulfate) increase the hydrophilic properties of PNIPAM through internal electrostatic repulsion which expands the polymer network and increases the LCST.¹⁵ Changing the monomer structure from *N*-isopropylacrylamide to *N*-isopropylmethacrylamide¹⁶⁻¹⁷ and *N*-cyclopropylacrylamide¹⁸ was found to change the LCST of the polymer from ~ 32 °C to ~ 45 °C and ~ 47 °C, respectively (Fig. 2.2).

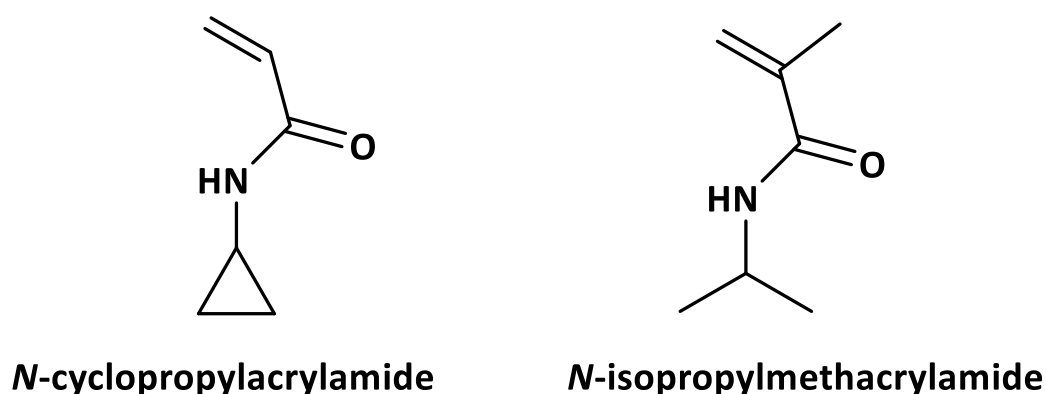


Fig. 2.2. Structure of *N*-isopropylmethacrylamide and *N*-cyclopropylacrylamide.

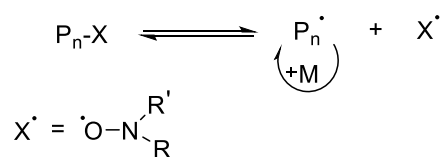
Kuramoto *et. al.* reported some copolymers of *N*-cyclopropylacrylamide with vinylferrocene with tuneable LCST (26°C – 47°C) depending on ferrocene content in the copolymers.¹⁸ Some studies also suggested to use a mixture of polymers (known as polymer blends) such as poly(methyl methacrylate)-poly(styrene-co-acrylonitrile) to tune the LCST of the system.¹⁹ For our studies, we decided to synthesize different chain length poly(*N*-isopropylmethacrylamide) (PNIPMAM) to check the effect of molecular weight on polymer LCST. Our preliminary results using PNIPAM (10 kDa) for protein delivery applications were promising³ but the phase transition temperature was too low for biological applications. Therefore, this chapter is focussed on the synthesis of PNIPMAM (≥ 10 kDa) with tuneable LCST.

2.2. Synthesis of thermo-responsive polyacrylamides

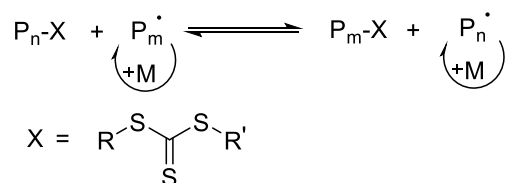
2.2.1. Radical polymerization

Polyacrylamides are usually synthesised by radical or anionic polymerisation. The electron-withdrawing nature of amide groups in acrylamides provide the monomers with potential reactivity favourable for anionic polymerization. However, the presence of acidic hydrogen in acrylamide itself and monosubstituted acrylamide inhibits the anionic vinyl polymerization.⁵⁵ Radical polymerization has been so far the most widely adapted method to obtain the polyacrylamides. In radical polymerisation, the polymer chains form after the successive addition of free radicals at the end of the growing chain. There are two types of radical polymerization; conventional (uncontrolled) and living (controlled), and, the main difference between these approaches is the termination step. In conventional polymerization,²⁷ the polymer chains are constantly active during propagation with uncontrolled termination. Therefore, the control over polydispersities, chain architecture and composition are somewhat restricted, which may generate undesirable polymers. Living polymerization works by having a reversible termination step based on establishing a rapid dynamic equilibration between growing polymer chain and the dormant species (**Scheme 2.1**). Undesirable chain termination through various active radical combinations is still possible, but the more likely cause of chain termination is the absence of monomer, which is suitable for generating polymers with a desired molecular weight and narrow distribution.

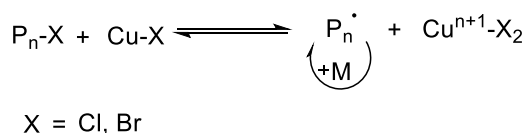
A) Dissociation-combination mechanism in NMP



B) Degenerate-transfer mechanism in RAFT



C) Atom transfer mechanism in ATRP



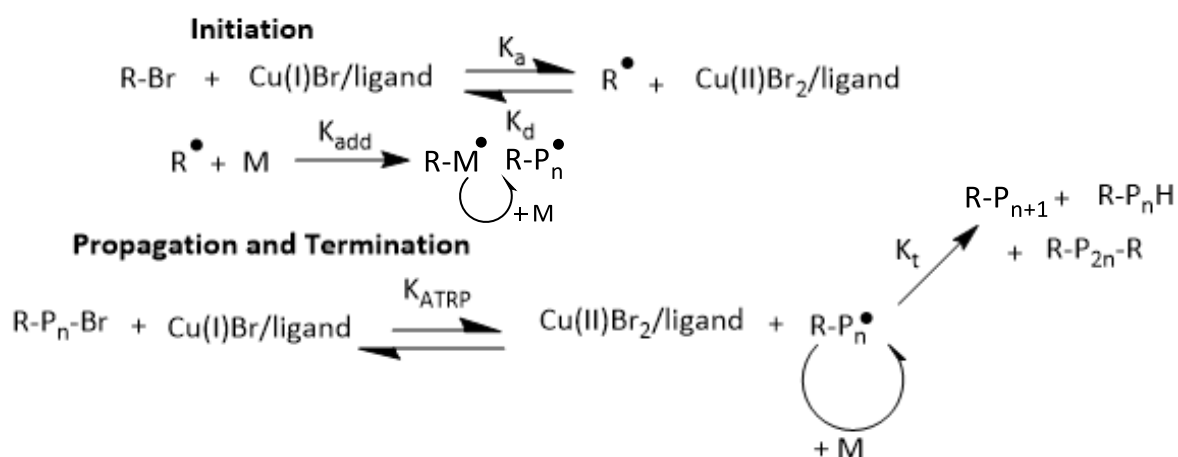
Scheme 2.1. Chain propagation mechanisms of commonly used living polymerization approaches: A) Dissociation-combination in NMP, B) Degenerate transfer in RAFT and C) Atom transfer in ATRP. Here, P_n represents dormant polymer, and $P_n^\cdot$ represents active, propagating polymer.

The synthesis of polyacrylamides has been reported using controlled free radical polymerization including atom transfer radical polymerization (ATRP),^{2,3,16-17} reversible addition fragmentation chain-transfer (RAFT) polymerization²⁰⁻²² and nitroxide mediated polymerization (NMP).²³⁻²⁴ NMP involves the use of an alkoxyamine initiator to generate polymers with well controlled stereochemistry and a low polydispersity index (PDI). However, the NMP requires high temperatures and lengthy polymerization times due to slow polymerization kinetics.²⁴ Additionally, its inability to control the polymerization of methacrylate monomers due to side reactions and/or slow recombination of the polymer radical with nitroxide is known. Hence, we didn't consider using NMP for PNIPMAM synthesis. On the other hand, due to their flexibility regarding the choice of end groups and catalytic species, RAFT and ATRP are well known living polymerization to obtain polyacrylamides with

a range of architectures and compositions. ATRP and RAFT polymerization are discussed in detail below.

2.2.1.1 Atom transfer radical polymerization (ATRP)

In ATRP, the radical deactivation occurs through reversible atom transfer. It involves a transition metal catalyst, such as copper, and an alkyl halide initiator. The initiation step involves the activation of the dormant species via one electron transfer from the transition metal complex which results in the radical generation. Oxidation of the transition metal complex is a reversible process in which the equilibrium largely favours the side with low radical concentrations. Number of polymer chains are regulated by the number of initiators formed, where each propagating chain has equal probability of reacting with monomer units to elongate the living polymer chains. This results in generating polymers with controlled molecular weights with narrow size distribution. A typical depiction of an ATRP can be seen in **Scheme 2.2**.



Scheme 2.2. Mechanism of an ATRP reaction using a bromine-based halide alkyl initiator and copper bromide-based transition metal catalysts: Here, R-Br is bromide-based radical initiator, Cu(I)Br/ligand are copper bromide-based transition metal/ligand complexes, R• is a generated radical, M is the monomer, R-P_n-Br is the dormant polymer species and R-P_n• is the active, propagating polymer species.

The initiation step in ATRP is a reversible process, which is determined by the radical concentrations, and hence, controls the rate of polymerization. The initiator ratio determines the number of polymer chains and a rapid initiation leads to a narrow weight distribution due to consistent chain propagation. In addition to this, an increase in the monomer ratio to initiator should increase the molecular weight of the polymer. Many researchers reported similar trends of increased molecular weights with increase in the monomer ratio using ATRP.^{3,16-17}

2.2.1.2. RAFT polymerization

Raft polymerization is a robust and versatile process, applicable to the majority of monomers. With the appropriate selection of the RAFT agent for the monomers and reaction conditions, it is possible to synthesize polymers with desired molecular mass and other properties.³³ The process of the RAFT polymerization is shown in **Fig. 2.3**.

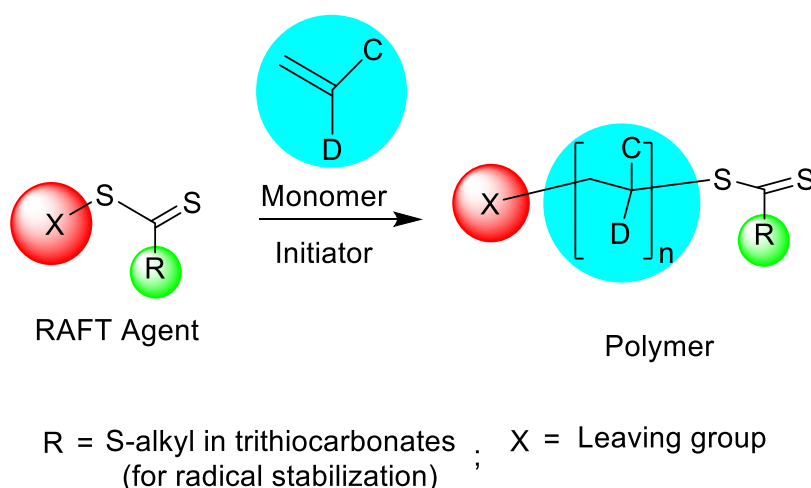
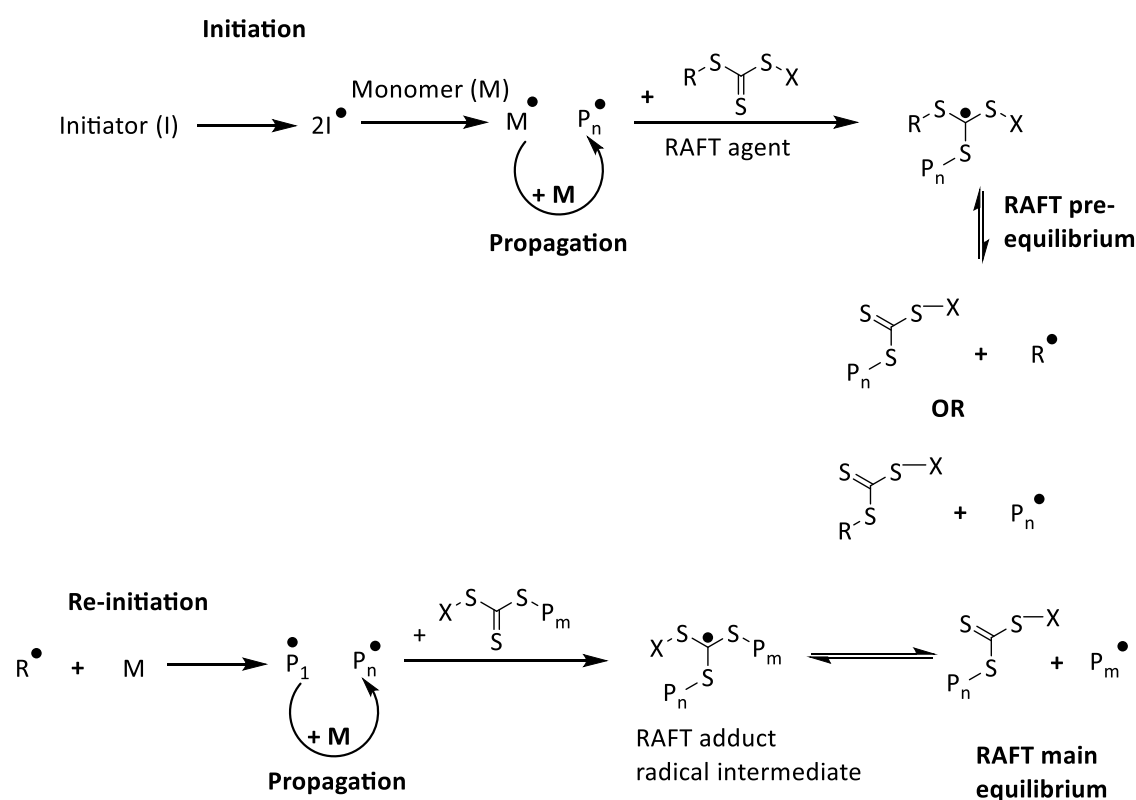


Fig. 2.3. RAFT polymerization process.

The RAFT polymerization reaction is started by generating radicals from a free radical initiator (mostly AIBN) followed by propagation. In the **Scheme 2.3**, the initiator decomposes to form two fragments ($2I^{\bullet}$) which react with a single monomer molecule to yield a propagating polymeric radical ($M^{\bullet}$). This polymeric radical after propagation reacts with the RAFT agent to form a RAFT adduct radical. This is a reversible step (RAFT pre-equilibrium) in which the intermediate RAFT adduct radical is capable of losing either the R group ($R^{\bullet}$) or the polymeric

species ($P_n^\bullet$). The leaving group radical ($R^\bullet$) then reacts with another monomer species, starting another active polymer chain. This is the most important part in the RAFT process (RAFT main equilibrium), in which, by a process of rapid interchange, the present radicals are shared among all species that have not yet undergone termination ($P_n^\bullet$ and $S=C(R)S-P_n$). Ideally the radicals are shared equally, causing chains to have equal opportunities for growth and a narrow molecular weight distribution.

The most important step in performing RAFT polymerization is the selection of an appropriate RAFT agent. The overall effectiveness of a RAFT agent depends on the properties of the homolytic leaving group (X) and the activating group (R). A wide range of thiocarbonylthio compounds has been reported for use as RAFT agent (**Fig. 2.4**).



Scheme 2. 3. Mechanism of a RAFT polymerization using an initiator and RAFT agent

For the polymerization of methacrylamides, aromatic dithioesters (**Fig. 2.4 (1-4)**) were reported amongst the most active RAFT agents.³⁴ However, these dithioesters are more prone to side reactions and hydrolysis, which could increase the polydispersity of the polymer. On

the other hand, trithiocarbonates (**Fig. 2.4 (9-10)**) provide a better balance between activity and prevalence of side reactions, and hence, currently are most popular RAFT agents for methacrylamide polymerization.³⁵⁻³⁶

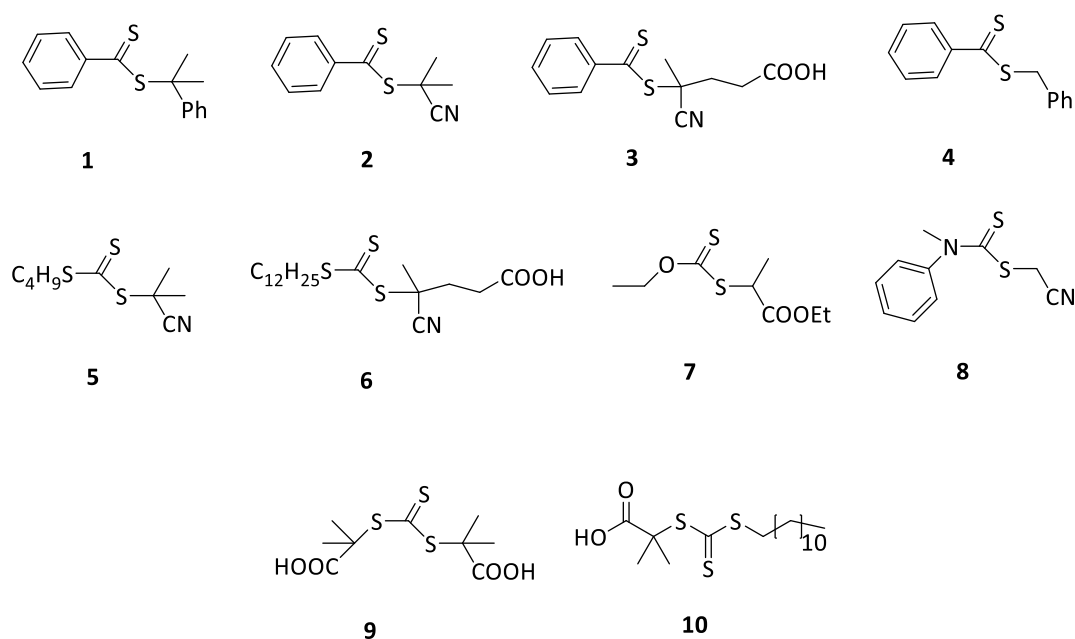


Fig. 2.4. Some common thiocarbonylthio based RAFT chain transfer agents.

We used both ATRP and RAFT to synthesize PNIPMAM. Although ATRP is not the best method to make poly-PNIPMAM, we did observe polymer formation very early on, and hoped that some reaction optimisation could improve yield/ M_w . This is why we did not change the method until after the optimisation attempts where we managed to improve PNIPMAM yield but not the M_w . RAFT polymerization was then used to synthesize PNIPMAM with a better control of M_w and polydispersity. In the next sections, synthesis and characterization of PNIPMAM synthesized via ATRP and RAFT polymerization have been discussed in detail.

2.2.2. Direct synthesis of PNIPMAM from NIPMAM using ATRP

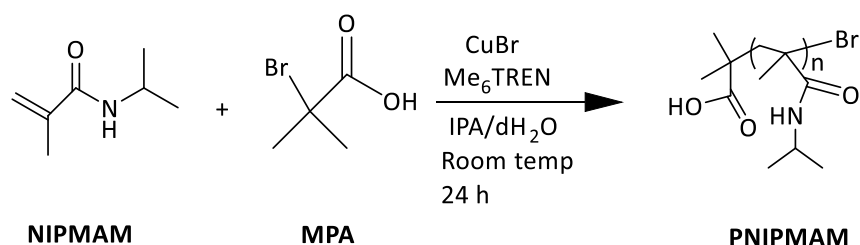
There were some successful reports on the direct conversion of monomer to PNIPAM using ATRP.^{2-3,25} PNIPMAM was synthesized via ATRP²⁵ using 2-methyl-2-bromoisobutyrate (MPA) as initiator and Cu(I)Br as catalyst (**Scheme 2.4**).

There was a successful formation of PNIPMAM (confirmed by $^1\text{H-NMR}$), but, the yield (6%) and the average molecular weight (M_w) were quite low (~ 3.5 kDa (MALDI-MS, **Section 2.4.3.2**)). It was due to the low conversion of the monomer to PNIPMAM. However, the LCST of PNIPMAM (50:1 NIPMAM:MPA) was ~ 51.7 °C (NanoDSF, **Section 2.4.4, table 2.9**). Therefore, we decided to explore reaction optimization to increase the yield as well as to tune PNIPMAM M_w . We used MALDI-MS as a quick tool to check the polymer mass distribution (**Section 2.4.3.2**). M_w determination is discussed in detail in later section.

In ATRP, solvents were reported to play a significant role in the controlled polymerization of acrylamides. Solvents should provide good solubility of the monomer and the catalytic system, and, will also affect the kinetics of the reaction. Xia *et. al.* reported the synthesis of different molecular weight PNIPAM in alcohols where branched alcohol isopropanol (IPA) gave high conversion and narrow molecular weight distribution.² In our case, the solubility of the reaction mixture improved significantly in the IPA. We tried a polymerization with increased monomer concentration from 10 wt % to 33 wt % where, both yield (15 %) and M_w (~ 4 kDa) of PNIPMAM improved. There were some reports in the literature on the effect of the monomer concentration on the polymerization where higher concentrations were found favourable for higher monomer conversion.^{2,16-17,26}

Alkyl halides (R-X , $\text{X} = \text{Cl, Br}$) are the commonly used initiators in the ATRP. Since the R-X bond cleavage would be easier in alkyl bromides as compared to alkyl chlorides, we used 2-methyl-2-bromopropionic acid (MPA) as an initiator.^{2,26} However, there were some reports where, researchers found it difficult to polymerize methacrylamides using alkyl bromides as initiators. Matyjaszewski *et. al.* reported that the ATRP of N,N -dimethylacrylamide (DMA) suffered from (a) deactivation of the copper catalyst through complexation with amide groups, (b) displacement of the terminal halide atom by amide groups, and (c) low values of the ATRP equilibrium constant.²⁸ Therefore, we tried R-Cl (2-methyl-2-chloropropionic acid (MCP)) as initiator and $\text{CuCl/Me}_6\text{TREN}$ as catalytic system,^{2,28} but there was no improvement in the yield and the weight distribution of PNIPMAM. $\text{CuCl/Me}_6\text{TREN}$ was reported to have many side reactions leading to an early termination, which could be the reason for low M_w PNIPMAM.²⁸ Therefore, the best catalytic system for PNIPMAM synthesis was $\text{CuBr/Me}_6\text{TREN}$ with MPA as radical initiator.

Reaction system polarity and monomer concentrations were found to affect the polymerization significantly. There were some reports where researchers used a mixture of polar solvents in order to achieve the optimal polarity suitable for the polymerization.^{3,25} We also tried various compositions of water and IPA for the polymerization where, the solubility of the reaction mixture improved. We performed a polymerization in high monomer concentration (50 wt%) with 1:10 IPA:water, and the PNIPMAM yield improved significantly (50 %). Hence, the optimized polymerization conditions were 50 wt% monomer concentration in 1:10 IPA:water as solvent, CuBr/Me₆TREN as catalyst and, MPA as radical initiator at room temperature for 24 h (**Scheme 2.5**).



Scheme 2.5. Reaction Scheme 2. for PNIPMAM synthesis. (Reaction conditions: 50:1:1:2 (Monomer:Catalyst:Initiator:Ligand), 5 g / 10 ml solvent)), MPA = 2-methyl-2-bromopropionic acid and Me₆TREN = tris[2-(dimethylamino)ethyl]amine.

Since ATRP is a living polymerization, if we increase the monomer concentration with respect to the initiator, there will be more monomer available for the same number of initiator chains result in the increased polymer chain length. We tried increasing monomer to initiator ratio from 50:1 to 100:1, but no increase in the weight distribution was observed. This could be due to an early termination of polymerization by removal of the bromine end group or the deactivation of the catalyst system with acrylamides or any side reaction leading to an early termination.^{16,26} There are some reports on the catalyst poisoning by the solvent or solvent-assisted side reactions, such as elimination of HX, which were found more pronounced in the polar solvents.³⁰⁻³¹ Altogether, there could be a number of possible reasons for not getting PNIPMAM with desired M_w (≥ 10 kDa) and hence a new approach was required.

Rathfon *et. al.* synthesized different molecular weight PNIPMAM, having LCST between 35 °C – 60 °C using a post functionalization approach of activated esters.¹⁶ An active ester *N*-succinimidyl methacrylate (MASI) was polymerised to poly(*N*-succinimidyl methacrylate) (PMASI) first, and the polymer was then reacted with an amine to convert the active ester groups into amides. Hence, it was decided to use this post functionalization approach of activated esters to synthesize PNIPMAM.

Table 2.1. Various optimization reactions in the attempt to synthesize different chain length PNIPMAM by a direct polymerization (ATRP) of NIPMAM monomer. Here, [M]: [I]: [C]: [L] = Monomer: Catalyst: Initiator: Ligand.

Sr. No.	[M]:[I]:[C]:[L]	Solvent	Monomer concentration (wt%)	Yield (%)	Theoretical mass (kDa)	Average molecular Mass, M_w (MALDI, kDa)
1	50:1:1:2	MeOH + dH ₂ O (1:10)	10 wt%	6	6.5	~ 3.5
2	50:1:1:2	IPA	33 wt%	15	6.5	~ 4.0
3	50:1:1:2 ^{a*}	IPA	33 wt%	10	6.5	~ 3.0
4	50:1:1:2	dH ₂ O + IPA (1:10)	50 wt%	10	6.5	~ 3.5
5	50:1:1:2	IPA + dH ₂ O (1:10)	50 wt%	50	6.5	~ 4.0
6	100:1:1:2	IPA + dH ₂ O (1:10)	50 wt%	40	12.8	~ 4.0

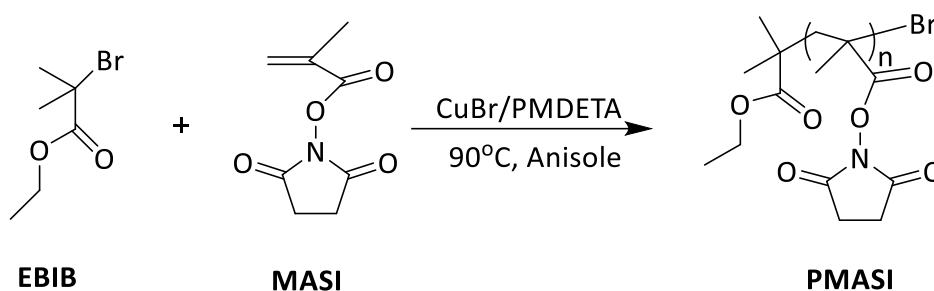
Notes. All reactions are performed at RT for 24 h with CuBr/Me₆TREN catalyst and MPA initiator (except for a* = CuCl/Me₆TREN catalyst and MCP initiator). Theoretical mass

calculated using formula: $M_{n,th} = M_{Initiator} + \frac{[monomer]_0}{[Initiator]_0} * conversion(\%) * M_{Repeat}$. Here, M_{Repeat} = mass of the repeat unit.

2.2.3. Synthesis of PNIPMAM using post-functionalization approach

2.2.3.1. Synthesis and characterisation of PMASI

To avoid the known problematic controlled polymerization of the acrylamides, MASI was used to synthesize PMASI polymer via ATRP (**Scheme 2.6**). The active ester polymer can then be reacted with isopropylamine (IPAm) to yield target PNIPMAM.



Scheme 2.6. Reaction Scheme for MASI monomer synthesis

The PMASI polymer was characterized by $^1\text{H-NMR}$ (DMSO-d_6) where a distinct broad multiplet corresponding to the MASI group (**1**) was observed between 2.6 – 3.0 ppm along with another broad peak between 1 - 1.8 ppm (**2 – 3**) corresponding to the protons present along the hydrocarbon polymer backbone (**Fig. 2.6**).

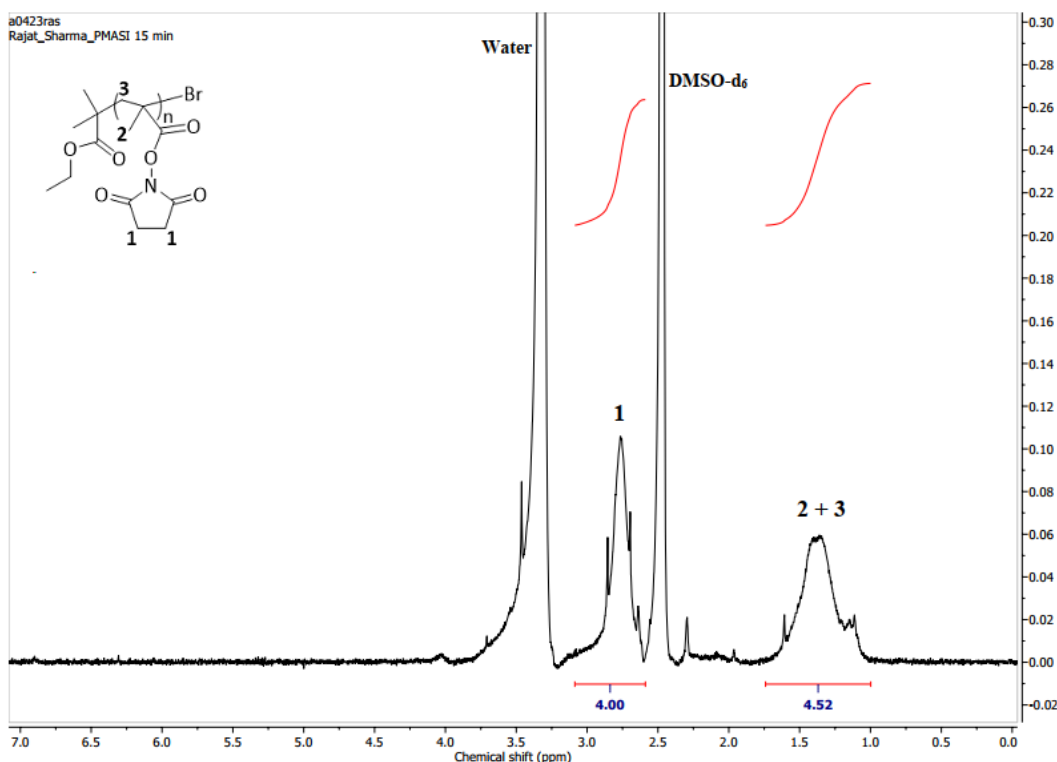


Fig. 2.6. ^1H -NMR of homopolymer PMASI in DMSO-d_6 .

To optimise the synthesis, we altered various components such as reaction time, concentration, temperature and monomer to initiator ratio. Changing monomer to initiator ratio could help us to get higher degree of polymerization and increased molecular weights of the PMASI. The results are displayed in the following table 2.2.

As expected, there was a significant increase in the yield (87 %) with increase in the monomer concentration from 12 wt% to 60 wt% (**Table 2.2**). There was a complete monomer conversion to PMASI within 15 min of the polymerization (^1H -NMR). However, there was no improvement in the M_w of the PMASI (~ 3.7 kDa). Since the reaction temperature was high (90 $^\circ\text{C}$), the reason for obtaining lower molecular weight polymers could be due to some side reactions leading to termination.²⁶ In order to slow down those termination processes, a polymerization at 50 $^\circ\text{C}$ was performed, where, a significant drop in PMASI yield (20 %) was observed. Hence, the optimized reaction conditions were 60 wt% monomer concentration in anisole, $\text{CuBr}/\text{PMDETA}$ catalyst system and EBIB as radical initiator at 90 $^\circ\text{C}$ for 15 min.

Table 2.2. Conditions and results for the copper-catalysed homo-polymerization of MASl monomer in anisole (scale = 2 g).

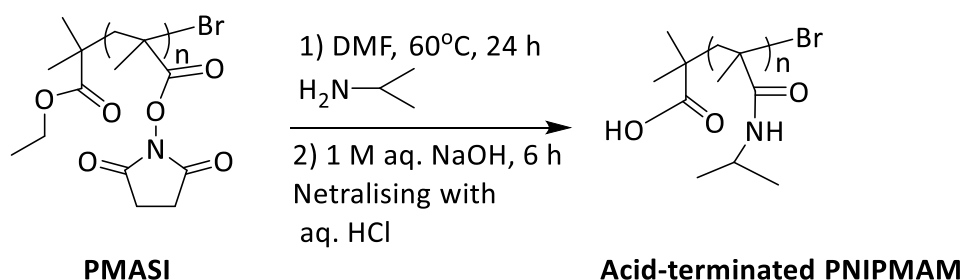
Sr. No.	[M]:[I]:[C]:[L] M = monomer, I = initiator C = catalyst L = Ligand	Time	Solvent's amount (ml)	Reaction temperature (°C)	Yield (%)	Theoretical mass (kDa)	Average molecular weight (MALDI, kDa)
1	50:1:1:1	1 h	16	90	52	9.3	~ 3.7
2	100:1:1:1	1 h	16	90	37	18.5	~ 2.0
3	50:1:1:2	15 min	3	90	87	9.3	~ 3.7
4	50:1:1:2	15 min	3	50	20	9.3	~ 2.7

Note. Theoretical mass calculated using formula: $M_{n,th} = M_{Initiator} + \frac{[monomer]_0}{[Initiator]_0} * conversion(\%) * M_{Repeat}$. Here, M_{Repeat} = mass of the repeat unit.

In order to determine the polymer LCST and hence decide whether the M_w is appropriate for our purposes, PMASl was converted to PNIPMAM, which is described in the next section.

2.2.3.2. Synthesis and characterisation of acid-terminated PNIPMAM from PMASl

After PMASl synthesis, the polymer sample was functionalized to form acid-terminated PNIPMAM through reaction of activated ester functional group with IPAm followed by hydrolysis of the ester end group (**Scheme 2.7**).



Scheme 2.7. Synthesis of Acid-terminated PNIPMAM.

Synthesis was done according to a reported procedure,¹⁶ which involved the reaction of PMASI with excess IPAm. The post-functionalization of PMASI to PNIPMAM was confirmed by ^1H NMR and ATR-FTIR. In ^1H NMR (D_2O), the complete disappearance of MASI group peak (2.79 ppm) and simultaneous appearance of peak corresponding to N-H amide singlet (**3**) confirmed the conversion of PMASI to PNIPMAM (**Fig. 2.7**). In addition, a large peak at ~ 1 ppm (**1**) and a broad peak between 3.8 – 4 ppm (**2**), corresponding to the i-Pr moiety were also seen.

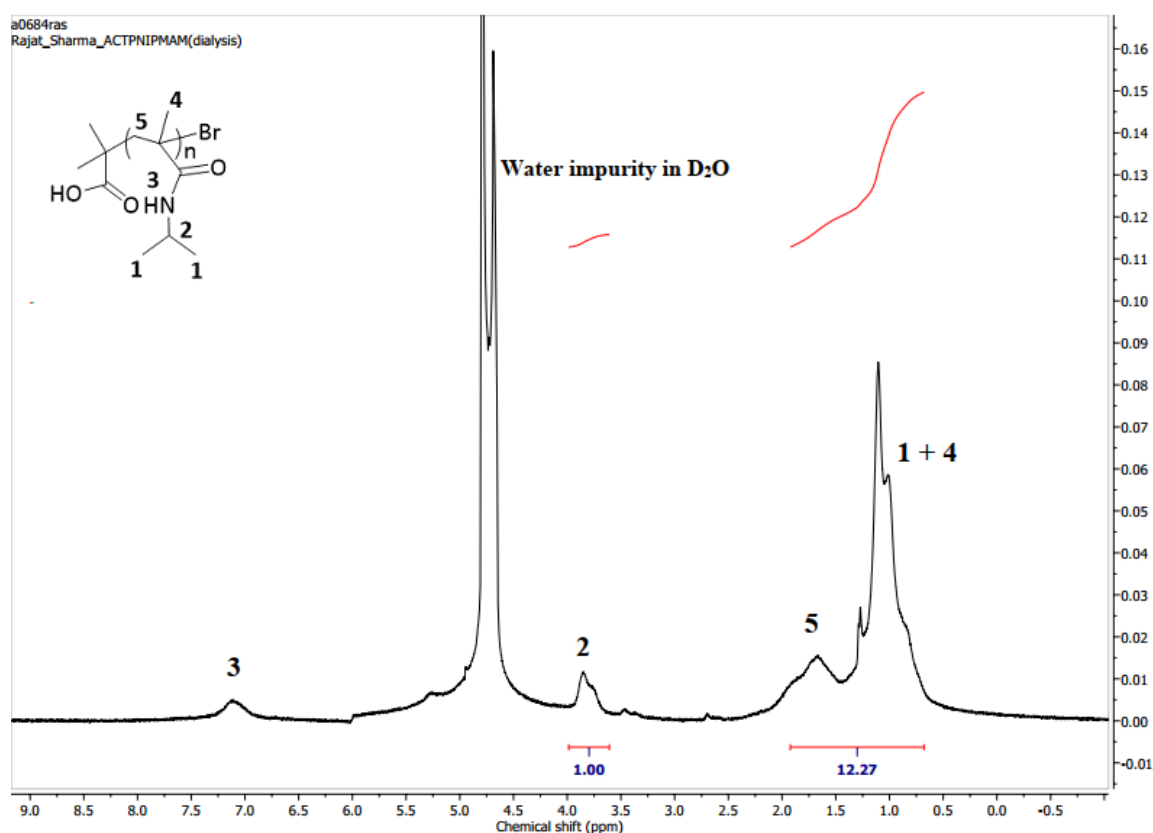


Fig. 2.7. ^1H -NMR of Acid-terminated PNIPMAM in D_2O .

The ATR-FTIR spectrum of PMASI and PNIPMAM (**Fig. 2.8**) also confirmed the successful conversion. The characteristic MASI bands (1808 , 1778 , 1732 cm^{-1}) were replaced with bands at 1630 and 1529 cm^{-1} , which was consistent with amide functionality present in the PNIPMAM polymer.¹⁷

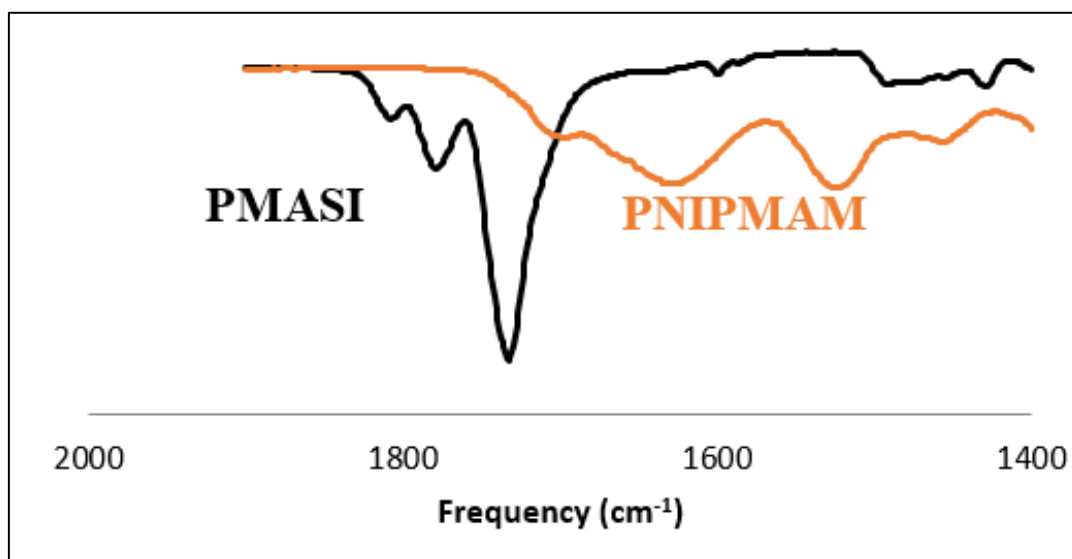
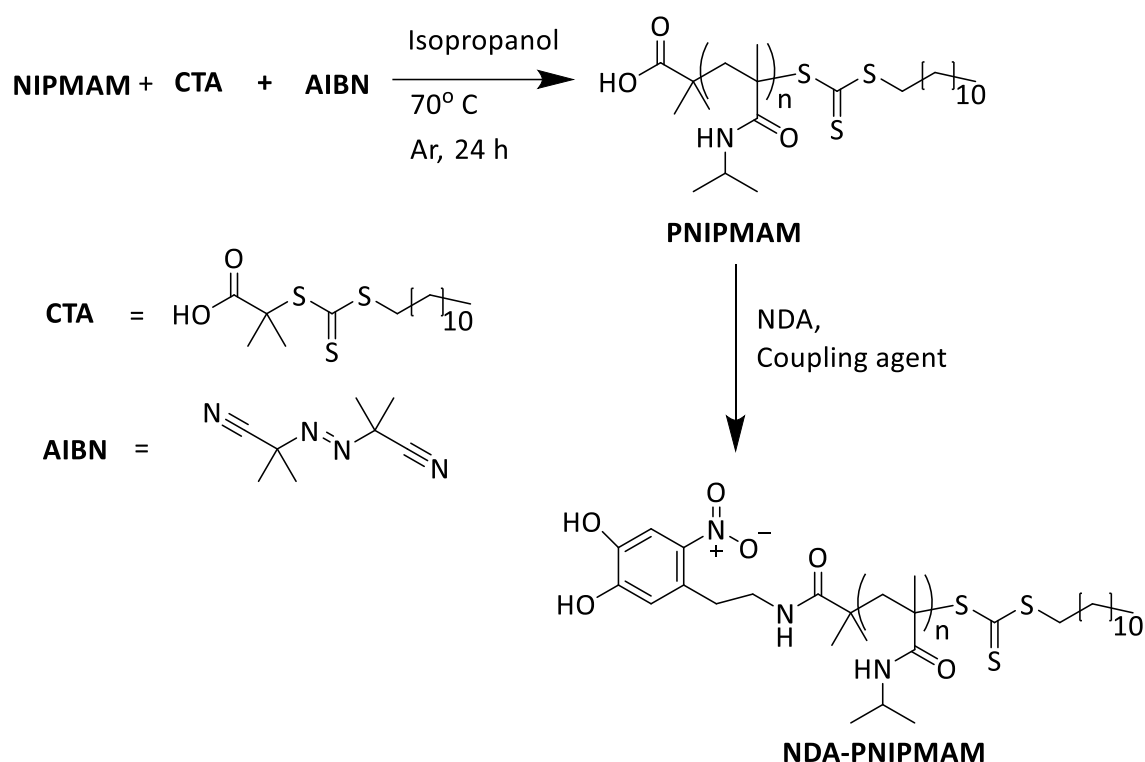


Fig. 2.8. FTIR spectra of PMASI and PNIPMAM.

Even though the formation of acid-terminated PNIPMAM was confirmed, no precipitation was observed upon heating the polymer solution to $70\text{ }^{\circ}\text{C}$. This broad molecular weight distribution, low M_n and no phase transition prompted us to change our strategy from ATRP to RAFT polymerization. Recently, RAFT polymerization has received greater attention due to its ability to polymerize highly functional monomers in a controlled fashion, and unlike ATRP, removal of a metal catalyst is not required.²⁰⁻²² It is known for a better control over molecular weight distributions, tolerance to the monomer structure and the nature of end groups as compared to ATRP. RAFT polymerisation is described in more detail in the next section.

2.2.4. RAFT polymerization to synthesize different chain length PNIPMAM

PNIPMAM was synthesized using RAFT polymerization of NIPMAM in isopropanol with azobisisobutyronitrile (AIBN) as a radical initiator and S-1-dodecyl-S'-(α,α' -dimethyl- α'' -acetic acid)trithiocarbonate as a chain transfer agent (CTA) (**Scheme 2.8**).



Scheme 2.8. RAFT polymerization and end group modification to give NDA end group PNIPMAM. (Reaction conditions: 100:1:0.2 (NIPMAM: CTA: AIBN), 1 g / 2 ml IPA)).

PNIPMAM was characterized by ^1H -NMR where the main peaks corresponding to the polymer repeat units were observed (**Fig. 2.9**). In the ^1H -NMR (D_2O) of the homo polymer, a broad peak at ~ 1 ppm (**1 + 4**) and a peak between 3.8 – 4 ppm (**2**) correspond to i-PrI moieties in the backbone. Amide proton peak at ~ 7 ppm (**3**) was also observed. M_w determination is discussed in a later section. The LCST of PNIPMAM (100:1 NIPMAM:CTA) was $\sim 50.5^\circ\text{C}$ (NanoDSF, **Section 2.4.4**, **Table 2.9**). A range of polymers with tuned LCST (43 - 51°C) were then synthesized using different NIPMAM:CTA ratio (100:1, 200:1, 300:1, 400:1 and 1000:1). It was

ideal to synthesize a range of different M_w PNIPMAM as it could help us understand protein-polymer interactions responsible for protein encapsulation/release.

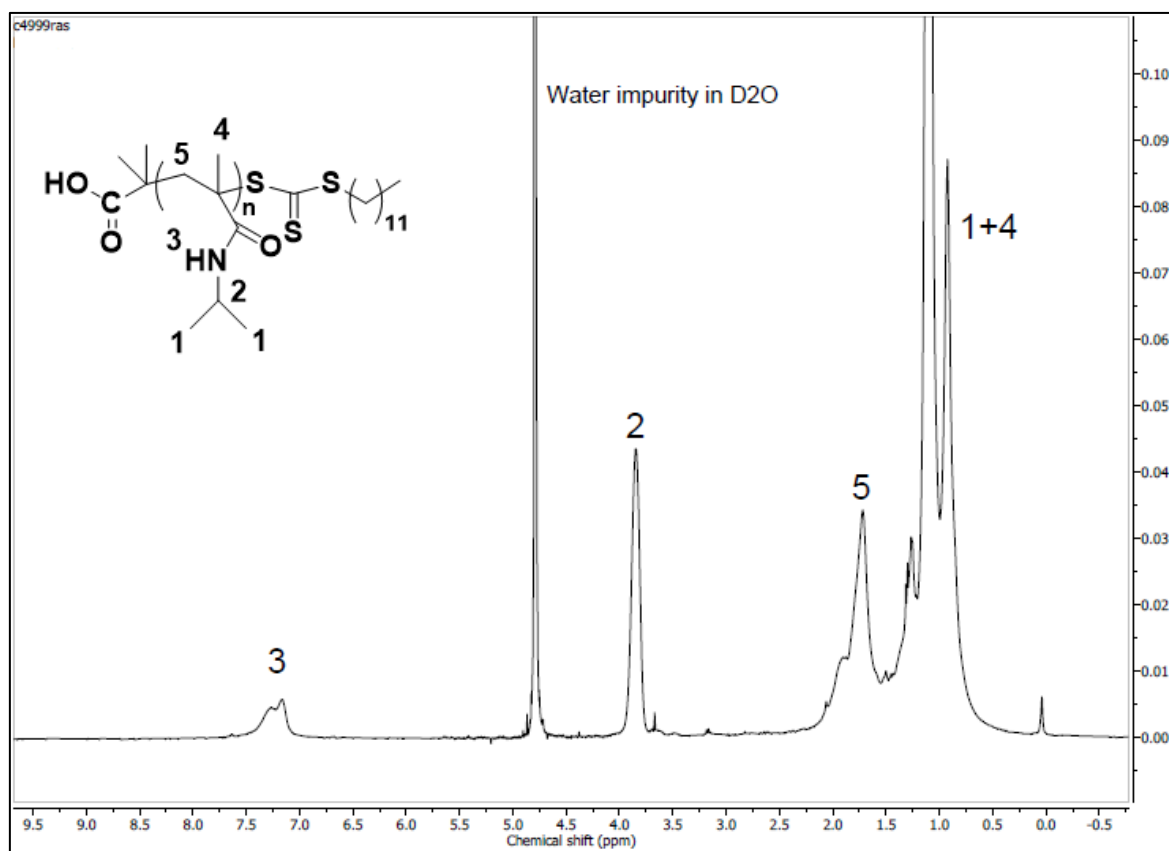


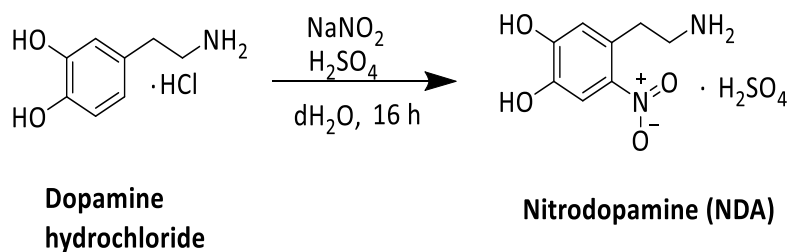
Fig. 2.9. ^1H -NMR of RAFT-PNIPMAM in D_2O .

After successful PNIPMAM synthesis, the next step was to develop a method to put the polymer onto the IONPs surface. Since catechol are known to have strong interactions with iron oxides, we decided to modify the carboxylic acid end group of PNIPMAM with catechol moieties. NDA is a known catechol anchor for iron oxide where, it can be coupled with the carboxylic acid end group of PNIPMAM either before (pre-functionalization) or after polymerisation (post-functionalization).³⁷⁻³⁹ We used post-functionalization approach because it allows incorporation of functionality that could be incompatible with the polymerization process.

In the next section, synthesis and characterization (^1H -NMR) of NDA and NDA-PNIPMAM are discussed.

2.3. Synthesis of NDA and NDA-PNIPMAM

NDA was synthesised by nitration of dopamine using sodium nitrite (**Scheme 2.9**).⁴⁰



Scheme 2.9. Synthesis route for 6-Nitrodopamine synthesis

NDA was characterized by ¹H NMR (**Fig. 2.10**), where two triplets corresponding to aliphatic CH₂ groups were detected at 3.05 (**2**) and 3.16 ppm (**1**), respectively. Peak at 6.87 (**3**) and 7.48 ppm (**4**) for CH groups of the aromatic ring also confirmed a successful NDA formation.

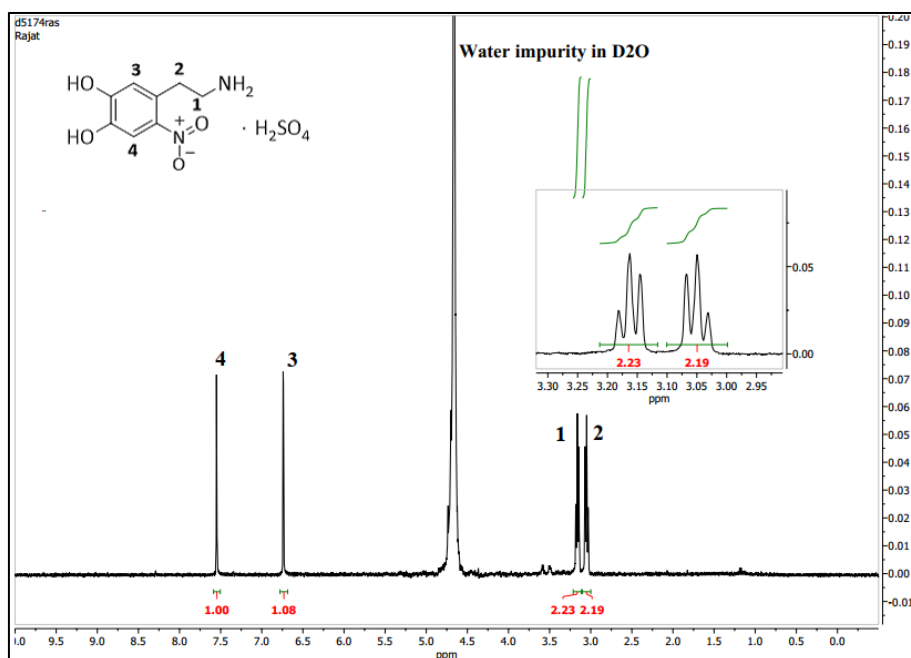


Fig. 2.10. ¹H-NMR of 6-Nitrodopamine in D₂O.

To facilitate the attachment of IONPs to the PNIPMAM, end group of the polymer was functionalized with NDA to obtain NDA-PNIPMAM. Functionalization with NDA was carried out by coupling the acid terminal group of the polymer to the amine functionality of NDA (**Scheme 2.5**). MALDI-MS was used as a confirmation where a complete disappearance of the residual polymer peak (alkene terminated) and the appearance of a new NDA functionalized polymer peaks was an indicator for a complete conversion of PNIPMAM to NDA-PNIPMAM (**Section 2.4.3.1**).

The method was a slight modification of a previous report on coupling NDA to PNIPAM.²⁵ We started with (1-cyano-2-ethoxy-2-oxoethylidenaminoxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) but could not observe a complete conversion of PNIPMAM to NDA-PNIPMAM in MALDI-MS. We then tried other coupling agents such as *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC.HCl), and *o*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HBTU) where, a complete conversion of PNIPMAM to NDA-PNIPMAM was obtained in the case of HBTU (**appendix A**). Hence, it was decided to use HBTU for coupling NDA to PNIPMAM.

In the next section, molecular weight characterization followed by phase transition studies of both RAFT and ATRP PNIPMAM are discussed.

2.4. Characterization: Molecular weight and phase transition of the PNIPMAM

2.4.1. Molecular weight characterization of RAFT-PNIPMAM

Polymer molecular weight indicates the average mass of the bulk polymer chains. All polymer molecules do not have exactly same molecular weight which results in a range or distribution of molecular weights. Hence, in order to check polymer dispersity, we calculate its average molecular weights (M_n , M_w) and polydispersity index (PDI). The number average molecular weight (M_n) is an average based on the number of polymer chains in a sample and is defined as follows (**equation 2.1**):

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \quad (2.1)$$

The weight average molecular weight (M_w), is the mass average with respect to the weight of each mass fraction and is calculated using the formula (**equation 2.2**).

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \quad (2.2)$$

Where N_i = number of molecules of a specific molecular weight and M_i = the specific molecular weight of those molecules.

PDI is a measure of the heterogeneity of the polymer chain lengths and is determined by dividing the M_w by the M_n . The measurement of a polymer's M_w is dependent on the total weight and total number of polymer particles present in its dilute solution, whereas M_n is dependent on the total number of polymer particles in its dilute solution regardless of polymer size (weight). As polydispersity should be fairly good in living polymerizations, the M_n and M_w of the polymer would be similar.

Gel permeation chromatography (GPC), known as size exclusion chromatography is commonly used to determine M_n of the polymers. In GPC, a dissolved polymer passes over a material (gel) held in a cylindrical column, which separates the polymer based on size. For our studies, the GPC was carried out using a set (PSS SDV High) of three analytical columns (300 x 8mm, particle diameter 5 μ m) of 1000, 1000000 and 10000000 Å pore sizes, made from styrene-divinylbenzene with copolymer network. Elution was with THF at 1 ml/min with a column temperature of 30 °C and detection was by refractive index. The retention time for the polymer was longer than for small molecules (toluene as reference) which suggested specific interactions between the polymer and the stationary phase. Some researchers claimed that GPC cannot be used to obtain molecular weight information for polyacrylamides due to filtration problems encountered before the analysis. They suggested the possibility of strong chain entanglement during filtration before entering GPC column.⁴¹⁻⁴² Here, in our results, it is possible that during filtration, the polymer chains got entangled and became bigger mass

units. Unfortunately, due to this reason, we could not use GPC to characterize PNIPMAM. Therefore, other characterization techniques were needed to get the information about the chain length of PNIPMAM.

It was difficult to characterize PNIPMAM end groups by ^1H -NMR since all end group protons would give peaks in the same region as methyl groups of the polymer (~ 1 ppm, **Fig. 2.9**). However, for NDA-PNIPMAM (**Fig. 2.11**), we did manage to see small peaks corresponding to the NDA functionalities in the aromatic region (**8, 9**). M_n of PNIPMAM (8.5 kDa) was calculated using the integration of these aromatic NDA protons relative to the single proton of the isopropyl moiety of the polymer (**2**). The molecular weight obtained was compared with the theoretical molecular weight (13 kDa), which was obtained using equation 2.3.²

$$M_{n,th} = M_{CTA} + \frac{[monomer]_0}{[CTA]_0} * conversion(\%) * M_{Repeat} \quad (2.3)$$

Here, $M_{n,th}$ is the theoretical molecular weight of the polymer, M_{CTA} is the mass of the chain transfer agent (364 g/mol), $\frac{[monomer]_0}{[CTA]_0}$ is the ratio of the initial monomer to CTA concentration (100:1), conversion(%) is the total conversion of monomer to polymer (100%) and M_{Repeat} is the mass of a repeat unit (127 g/mol).

NDA-PNIPMAM will have relatively low atomic ratio of NDA functionalities, which makes its accurate integration difficult ($M_w \leq 40$ kDa). This could be the reason for lower experimental M_n than theoretical M_n . Therefore, further characterization of polymer molecular weight was required, and the findings are reported in the next section.

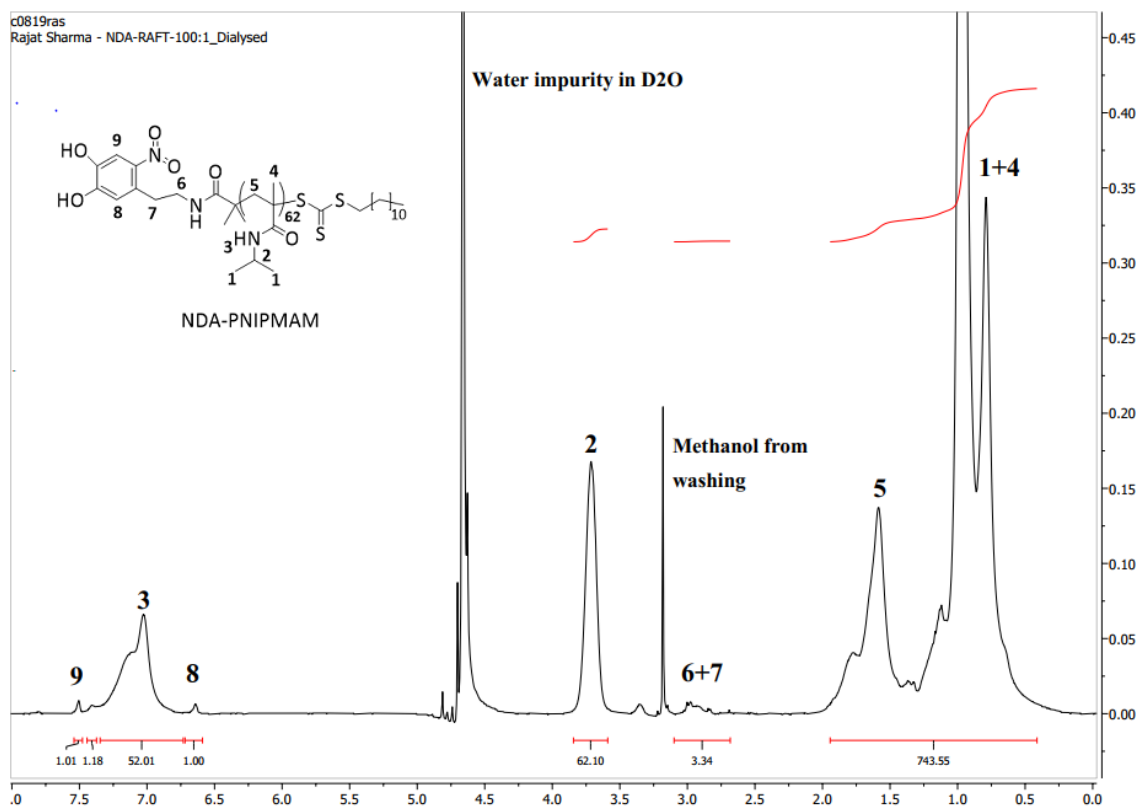


Fig. 2.11. $^1\text{H-NMR}$ of NDA-PNIPMAM in D_2O .

2.4.2. Determination of PNIPMAM molecular weight using UV-Vis spectroscopy

After the successful RAFT polymerization, the end group should remain intact where one side would contain carboxylic acid and other side would have thiocarbonylthio end groups. Molecular weight of polymer can be determined from the UV absorption of the $\pi\text{-}\pi^*$ band of these thiocarbonyl moieties (300–310 nm).⁴³ Fig. 2.12 showed the UV-Vis spectrum of RAFT-PNIPMAM in DCM.

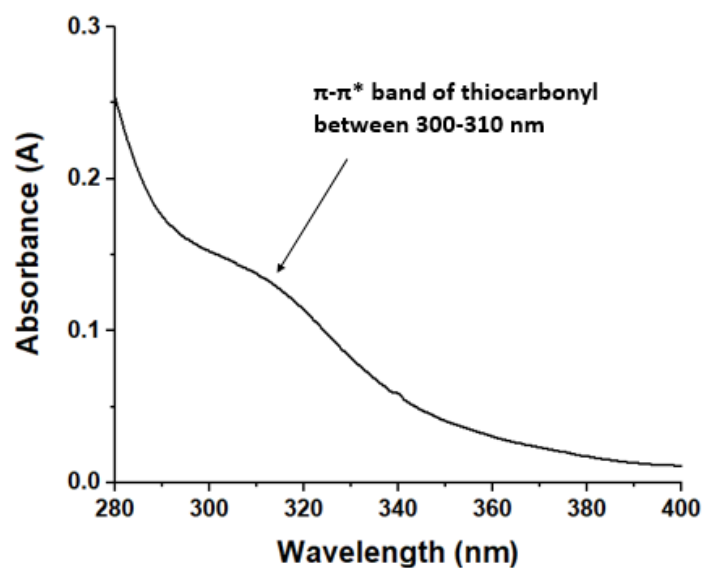


Fig. 2.12. UV-Vis spectrum of RAFT-PNIPMAM in DCM showing a shoulder peak corresponding to π - π^* band of the thiocarbonyl end group.

Presence of the peak corresponding to thiocarbonyl end group proved the successful RAFT synthesis. For the molecular weight calculation, molar extinction coefficient of chain transfer agent was calculated by using equation 2.4.

$$\epsilon = \frac{A}{c \cdot l} \quad (2.4)$$

Here, ϵ = molar extinction coefficient ($\text{mol}^{-1}\text{cm}^{-1}$), c = concentration of the CTA and l = path length (1 cm).

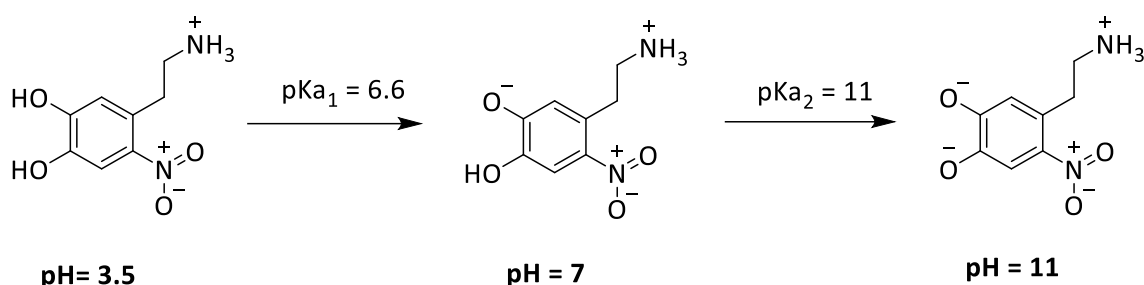
The molecular weight of the polymer was calculated by using following equation 2.5:

$$M_n = \frac{(m * \epsilon * l)}{A * V} \quad (2.5)$$

Where M_n = number average molecular weight of polymer, m = mass of the polymer taken for analysis and V = total volume of polymer solution.

The molar extinction coefficient of CTA was $10560.6 \text{ M}^{-1}\text{cm}^{-1}$ (in DCM, $\lambda_{\text{max}} = 308 \text{ nm}$).⁵¹ Molecular weight of the RAFT-PNIPMAM was calculated to be $\sim 16 \text{ kDa}$, which was in accordance with the theoretical weight (13 kDa). However, the thiocarbonyl peak is a shoulder and we couldn't separate it from the background. Therefore, the experimental M_n measurement using thiocarbonyl peak wasn't reliable.

Additionally, NDA moieties on PNIPMAM after coupling could also be monitored by UV-Vis spectroscopy. UV-Vis spectrum of free NDA gives a peak at 350 nm (λ_{max}) at acidic pH, which shifts to high wavelength at basic pH (**Scheme 2.10**). This bathochromic shift is due to possible resonance of hydroquinone groups of NDA at basic pH.



Scheme 2.10. Nitrodopamine at different pH (pH = 3.5, 7 and 11).

Due to this peak shift, a low pH spectrum can therefore be used for background correction of the high pH spectrum. Hence an appropriate pH range was selected (pH = 3.5 and 9), and, UV-Vis spectra of low M_n NDA-PNIPMAM ($\sim 8.5 \text{ kDa}$, $^1\text{H-NMR}$) were recorded, accordingly. At pH 3.5, both thiocarbonyl and NDA end groups gave broad UV absorption peaks (shoulders), which were difficult to distinguish and quantify. Expected behaviour from the NDA end groups of PNIPMAM was observed where, λ_{max} shifted from 350 nm to 422 nm with increase in the pH from 3.5 to 9, respectively. Additionally, peaks corresponding to both thiocarbonyl (308 nm) and NDA moieties (422 nm) at pH 9, confirmed the successful NDA coupling along with the preservation of thiocarbonyl end group (**Fig. 2.13**).

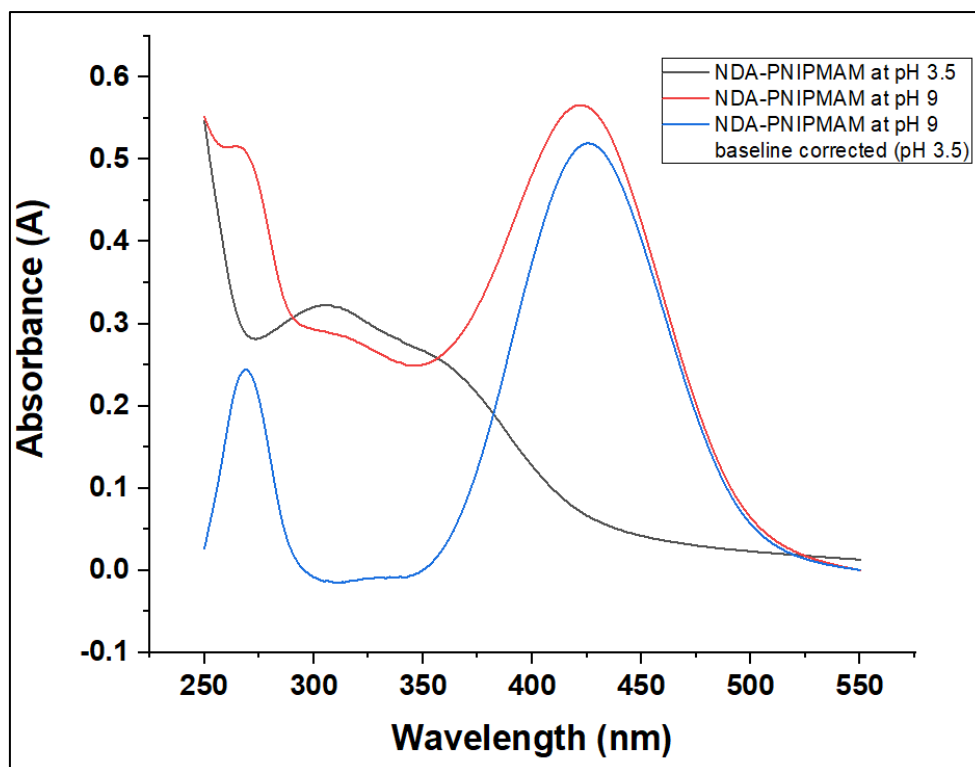


Fig. 2.13. UV-Vis spectroscopy analysis of RAFT-PNIPMAM at different pH system (pH = 3.5 and 9). Spectrum at pH = 3.5 is used to subtract polymer's background at pH 9.

The NDA peak at pH 9 was stronger than thiocarbonyl end group and hence could be used more accurately for determining polymer M_n . However, this assumes 100% NDA functionalisation of PNIPMAM, which was confirmed by MALDI-MS (**Section 2.4.3**) for the low M_n polymers (< 10 kDa). Another advantage of using NDA functionalities to determine polymer's M_n was; pH 3.5 spectrum could be used as a background for the pH 9 spectrum of NDA-PNIPMAM which improved the accuracy of M_n determination. The molar extinction coefficient of NDA was $9,600 \text{ mol}^{-1}\text{cm}^{-1}$ (**appendix B**). M_n of the RAFT-PNIPMAM calculated using NDA functionalities was $\sim 7.5 \text{ kDa}$ which was in accordance with NMR ($\sim 8.5 \text{ kDa}$). Therefore, it was decided to record UV-Vis spectra for different monomer to CTA ratio polymers synthesized using RAFT polymerization (100:1, 200:1, 300:1, 400:1 and 1000:1) (**Fig. 2.14**).

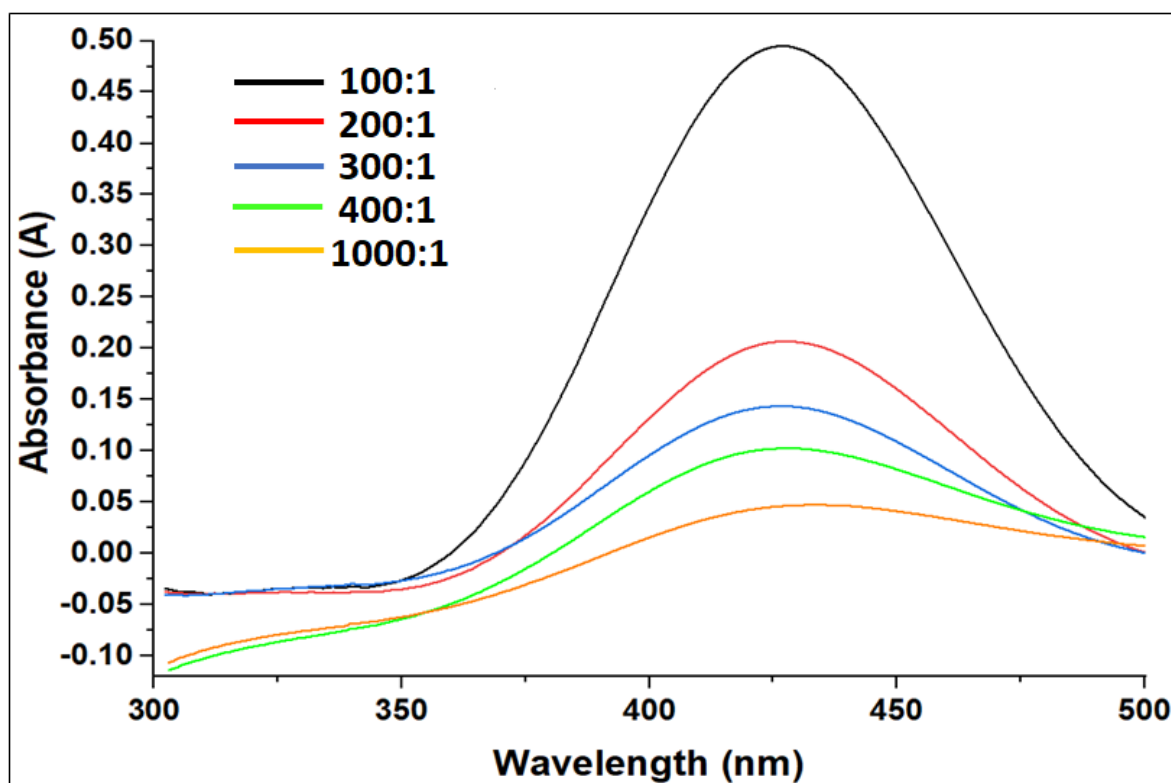


Fig. 2.14. UV-Vis spectra of various RAFT-PNIPMAM at pH 9 (pH 3.5 as baseline).

Recorded UV-Vis spectra were then used to characterize M_n of different PNIPMAM, and, to check the integrity of the polymer end groups.

Table 2.3. Different chain length PNIPMAM synthesized via RAFT polymerization.

Monomer: CTA	Yield (%)	Theoretical molecular weight (kDa)	Average molecular weight (M_n , kDa)	
			$^1\text{H-NMR}$	UV-Vis
100:1	70	~ 12.7	~ 8.5	~ 7.5
200:1	68	~ 25.4	~ 20	~ 19
300:1	72	~ 38	~ 28	~ 27
400:1	80	~ 50	~ 43	~ 40
1000:1	76	~ 127	--	~ 89

Consistent M_n using NMR and UV validates the UV method (**Table 2.3**) but there is still uncertainty about the polydispersity of bigger M_w PNIPMAM. Hence, another method to confirm molecular weight distribution was sought and the findings are discussed in the next section.

2.4.3. Molecular weight determination using MALDI-MS

MALDI-MS (matrix assisted laser desorption ionization - mass spectrometry) is a soft ionization process, which uses organic matrices to ionise large non-volatile molecules such as proteins, polysaccharides and synthetic polymers with minimum fragmentation. There were numerous reports on synthetic polymer characterization using MALDI-MS, where M_w and end group were successfully analysed.^{2-3,25} Any modification to the polymer end group would result in a different mass as compared to the starting polymer, which could precisely be measured using MALDI-MS. However, the accuracy of this method is difficult to assess due to the lack of suitable PNIPMAM standards of accurately known molecular weights and molecular weight distributions. There are several experimental and instrumental factors that may affect the determination of M_w using MALDI-MS (e.g. the sensitivity in different mass ranges is different and depends on instrument parameters).

In MALDI, a mixture of polymer with a suitable matrix compound is irradiated with a pulsed laser beam. Matrix absorbs the laser pulse (UV or IR laser) and indirectly ionizes the polymer molecules following energy transfer, which are detected by the detector. In this section, our attempts to characterize PNIPMAM M_w using MALDI-MS are discussed. The section is further divided into three subsections covering polymers characterised by RAFT, ATRP and PMASI.

2.4.3.1. Molecular weight characterization of RAFT-PNIPMAM using MALDI-MS

Molecular weight characterization using MALDI-MS involves two main steps: sample preparation and spectral analysis. Sample preparation involved finding a suitable matrix followed by developing a favourable sample spotting method to get detectable polymer ionization. We used trans-3-indolacrylic acid (IAA) as a matrix (dissolved in THF = 20 mg/ml) with the normal premix spotting method (premix matrix and polymer solutions before

spotting). The polymer was poorly soluble in THF and hence was dissolved in methanol (1 mg/ml) prior to premix spotting. To study the chemistry of PNIPMAM end group, short chain polymer (~ 8.5 kDa, $^1\text{H-NMR}$) was used due to its high signal to noise ratio and clean isotopic distribution in the spectrum. The background peaks due to IAA matrix were observed between 700 Da to 2.5 kDa and were removed using mass suppression at 3 kDa (**Fig. 2.15**). The M_w obtained using MALDI-MS was ~ 6 kDa which was comparable to NMR (~ 8.5 kDa) and UV method (~ 7.5 kDa).

Mass spectra obtained with MALDI-MS were used for the end group analysis of PNIPMAM and NDA-PNIPMAM. The theoretical isotopic distribution pattern was compared to the actual isotopic distribution corresponding to the predicted structure (shown in **Fig. 2.16, 2.17**).

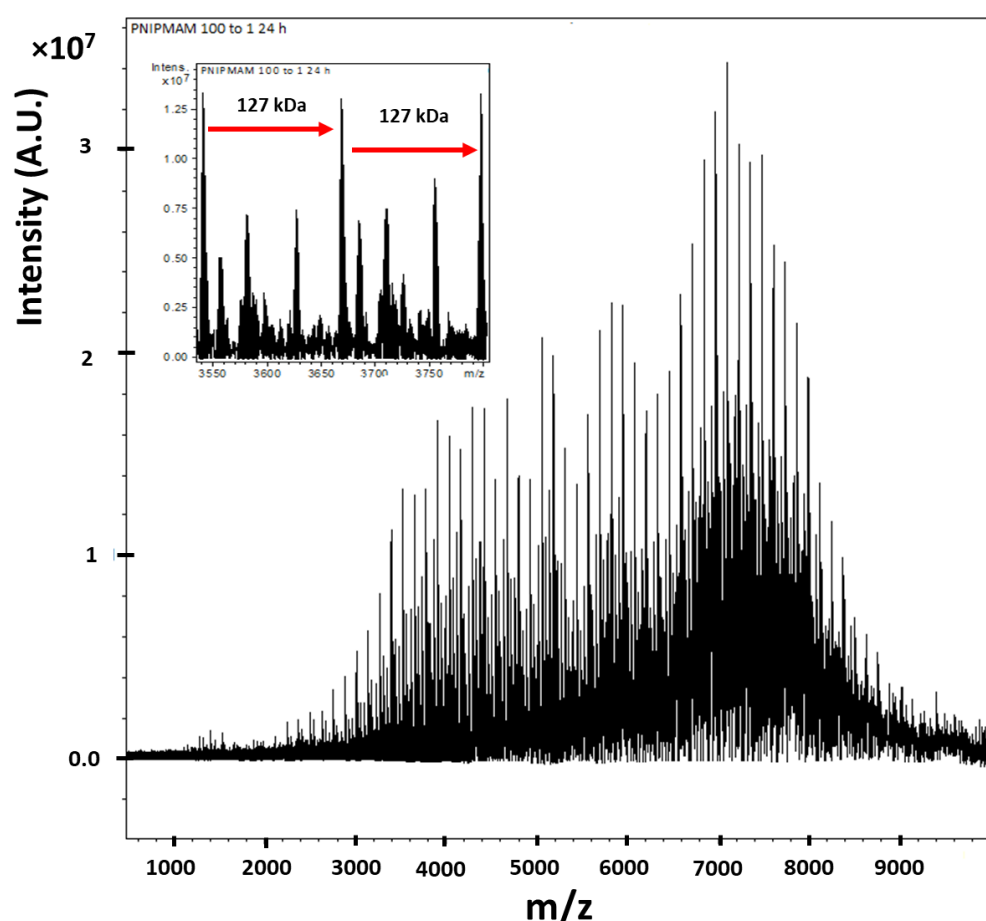


Fig. 2.15. MALDI-MS spectra showing the effect of using mass suppression. RAFT-PNIPMAM ($M_n = 5414.6$ Da, $M_w = 5914.7$ Da and Poly-dispersity index (M_w/M_n) = 1.09). Distance between repeat units were 127 Da.

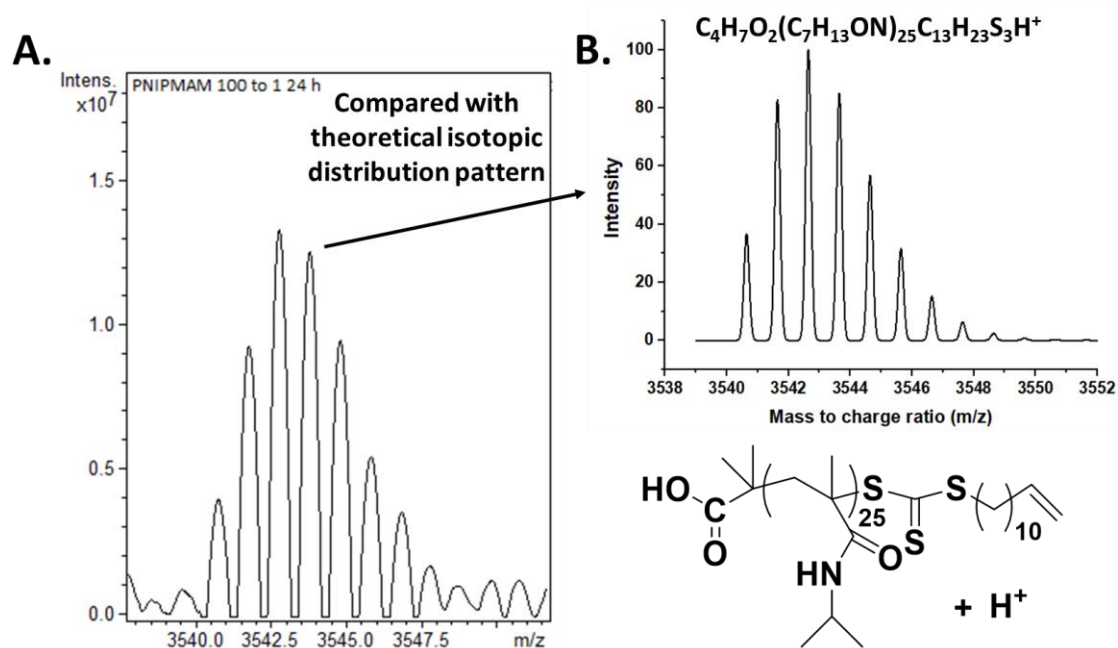


Fig. 2.16. End group analysis of the major peak in the MALDI-MS spectrum of PNIPMAM. A) Isotopic distribution pattern for the major peak contained mixture of peaks. B) Theoretical isotopic distribution was used to predict the structure of the molecular ionic species.

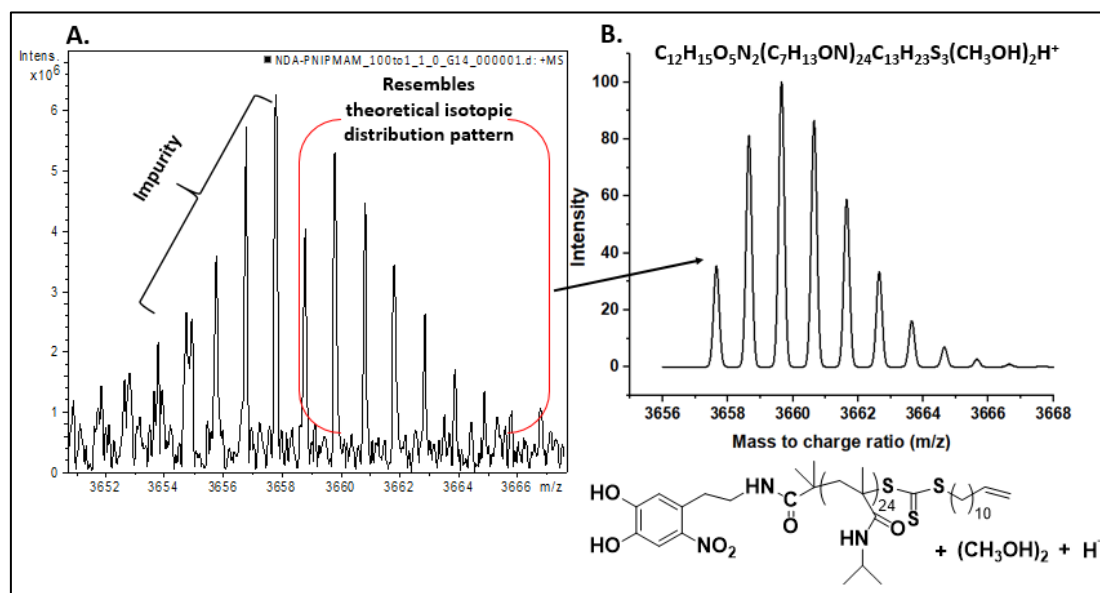


Fig. 2.17. End group analysis of the major peak in the MALDI-MS spectrum of NDA-PNIPMAM. A) Isotopic distribution pattern for the major peak contained mixture of peaks. B) Theoretical isotopic distribution was used to predict the structure of the molecular ionic species.

In the MALDI-MS spectrum of PNIPMAM, a set of 5 repeating peaks were obtained. The major peak corresponded to a dehydrogenated species with the molecular formula $C_4H_7O_2(C_7H_{13}ON)_{25}C_{13}H_{23}S_3H^+$ (3542.6 Da) (**Table 2.4**). The other peaks at $m/z = 3559.6$ and $m/z = 3582.7$ corresponded to the ionic species $C_4H_7O_2(C_7H_{13}ON)_{18}C_{13}H_{25}S_3NH_4^+$ and $C_4H_7O_2(C_7H_{13}ON)_{18}C_{13}H_{25}S_3K^+$, respectively (tentative). Peak at $m/z = 3600.6$ corresponded to a potassium+water adduct with the formula $C_4H_7O_2(C_7H_{13}ON)_{18}C_{13}H_{25}S_3K^+ \cdot H_2O$ (tentative). Final peak at $m/z = 3628.7$ corresponded to a methanol adduct with the formula $C_4H_7O_2(C_7H_{13}ON)_{18}C_{13}H_{25}S_3(CH_3OH)_2Na^+$ (tentative). Peaks 3 and 4 appeared to be hydrogenated.⁵²

End group analysis using MALDI-MS was used to confirm the complete conversion of PNIPMAM to NDA-PNIPMAM where polymer peaks were replaced completely by a new set of 4 peaks (**Fig. 2.18**).

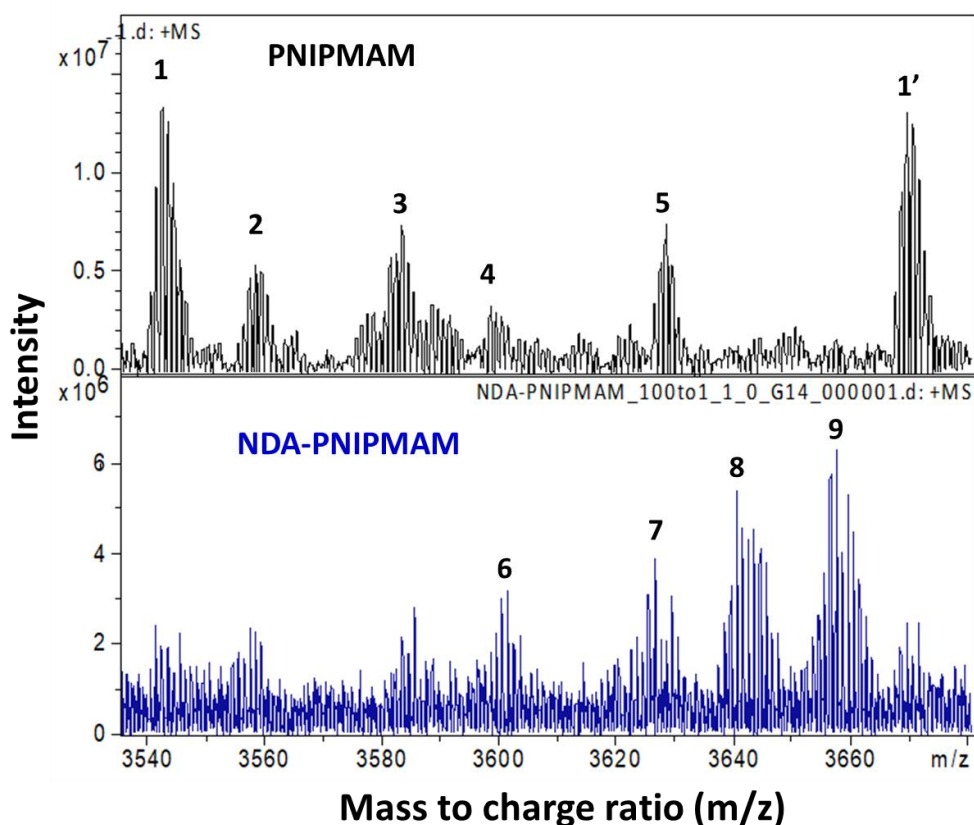


Fig. 2.18. MALDI-MS spectrum of PNIPMAM showing a set of 4 peaks replaced by a set of 4 new peaks in NDA-PNIPMAM confirmed the successful end group functionalization of PNIPMAM with NDA.

These four peaks in MALDI-MS of NDA-PNIPMAM were attributed to the chains bearing an NDA residue at one end and trithiocarbonate (from CTA) residue at the other end. The peak at $m/z = 3598.6$ corresponded to the molecular ion $C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}C_{13}H_{25}S_3H^+$ (**Table 2.5**). Since methanol was used to dissolve polymer during sample preparation, other 3 peaks were methanol adducts (tentative).⁵³ The major peak at $m/z = 3659.6$ was attributed to the molecular ion $C_{12}H_{13}O_5N_2(C_7H_{13}ON)_{24}C_{13}H_{25}S_3(CH_3OH)_2H^+$.

Table 2.4. Molecular formulas and predicted structures corresponding to the peaks obtained in the MALDI-MS of PNIPMAM (**Fig. 2.18**).

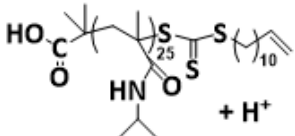
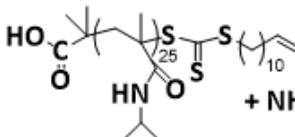
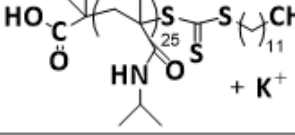
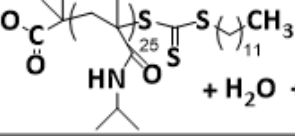
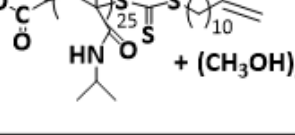
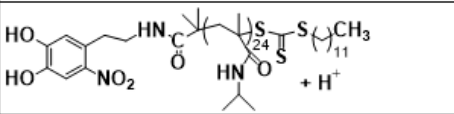
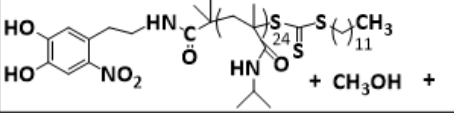
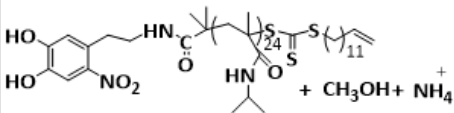
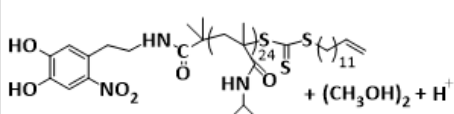
Peak No.	Molecular formula	Experimental m/z	Estimated m/z	Predicted structure
1	$C_4H_7O_2(C_7H_{13}ON)_{25}$ $C_{13}H_{23}S_3H^+$	3542.7	3542.6	
2	$C_4H_7O_2(C_7H_{13}ON)_{25}$ $C_{13}H_{23}S_3NH_4^+$	3559.6	3559.6	
3	$C_4H_7O_2(C_7H_{13}ON)_{25}$ $C_{13}H_{25}S_3K^+$	3582.7	3582.7	
4	$C_4H_7O_2(C_7H_{13}ON)_{25}$ $C_{13}H_{25}S_3H_2OK^+$	3600.6	3600.6	
5	$C_4H_7O_2(C_7H_{13}ON)_{25}$ $C_{13}H_{23}S_3(CH_3OH)_2Na^+$	3628.7	3628.6	

Table 2.5. Molecular formulas and predicted structures corresponding to the peaks obtained in the MALDI-MS of NDA-PNIPMAM (**Fig. 2.18**).

Peak No.	Molecular formula	Experimental m/z	Estimated m/z	Predicted structure
6	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}$ $C_{13}H_{25}S_3H^+$	3598.2	3597.6	
7	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}$ $C_{13}H_{25}S_3CH_3OHH^+$	3629.8	3629.6	
8	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}$ $C_{13}H_{23}S_3CH_3OHNH_4^+$	3644.5	3644.6	
9	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}$ $C_{13}H_{23}S_3(CH_3OH)_2H^+$	3659.6	3659.6	

After the successful end group analysis of short chain PNIPMAM and NDA-PNIPMAM, we tried to characterize higher molecular weight PNIPMAM (> 10 kDa, UV-Vis) using MALDI. In our case, only singly charged ions were observed. As multiply-charged ions are not present, detection is limited by the molecular weight detection range of available MALDI-MS instruments. The mass detection limit in the instrument for which we had optimized the conditions (matrix, spotting etc.) was 10 kDa. Therefore, for the polymers above this mass limit, we used a different instrument (Bruker Ultraflex), with upper detection limit of 75 kDa. With the previously optimized conditions, MS of RAFT-synthesised polymers showed very weak intensity spectra, and hence the MS conditions were re-optimised.

We started with using different matrices;⁵⁴ 2-(4-hydroxyphenylazo)benzoic acid(HABA), alpha-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), dithranol (DTH), sinapic acid (SA), 2-mercaptobenzothiazol (MBT) and trans-3-indolacrylic acid (IAA). Three spotting methods were used: 1) pre-mixing ((matrix (M) + sample (S)) spot), 2) sandwich method (M spot + S spot + M spot), and premixing sandwich ((M+S) spot, + S spot). For high molecular weight polymer (40 KDa (UV-Vis)), only HABA, MBT and IAA with premixing sandwich method gave some detectable ionization of PNIPMAM but the signals were quite weak (**Fig. 2.19**). Some of MALDI protocols in the literature suggested using ionic compounds

such as LiCl, AgCl etc. with the matrix to enhance the signals.⁴⁵ We also tried these ionic compounds namely NaTFA, LiCl and AgCl during the spotting, out of which LiCl gave us enhanced polymer signals. Data obtained was quite noisy and hence, we couldn't assess the average molecular weight of the polymer.

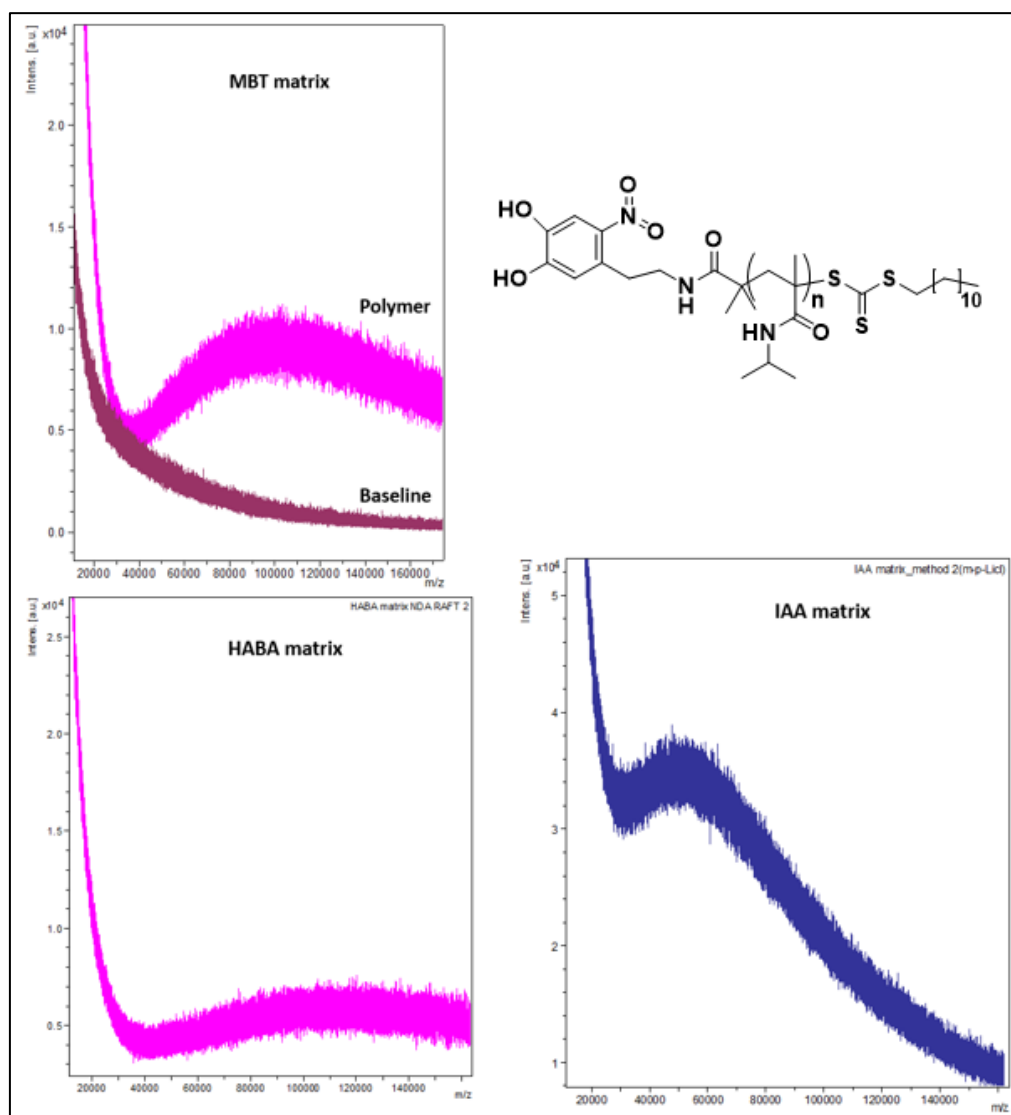


Fig. 2.19. Active matrices for the high molecular weight PNIPMAM in MALDI-MS. Linear positive ion mode with no mass suppression and high signal smoothing.

Since the MALDI-MS data obtained for high molecular weight PNIPMAM was noisy, instrument parameters were optimised to obtain the best signal to noise ratio. These matrices (MBT, HABA and IAA) were giving signals towards lower mass range (between 750 – 2000 Da), it was

saturating the detector and hence the polymer signals were poor. Therefore, the mass suppression at 30 kDa was used to avoid this saturation due to matrix signals. Moreover, to increase the sensitivity of the instrument, medium signal smoothing was used to detect more signals for the higher molecular weight polymer. Satisfactory MS spectra in the expected mass range (UV-Vis) were obtained with IAA matrix system for higher molecular weight polymers (400:1 (40 kDa, UV-Vis) and 1000:1 (89 kDa, UV-Vis)) (**Fig. 2.20**). However, the MS resolution wasn't sufficient for accurately predicting M_w and thus PDI of these high M_w polymers.

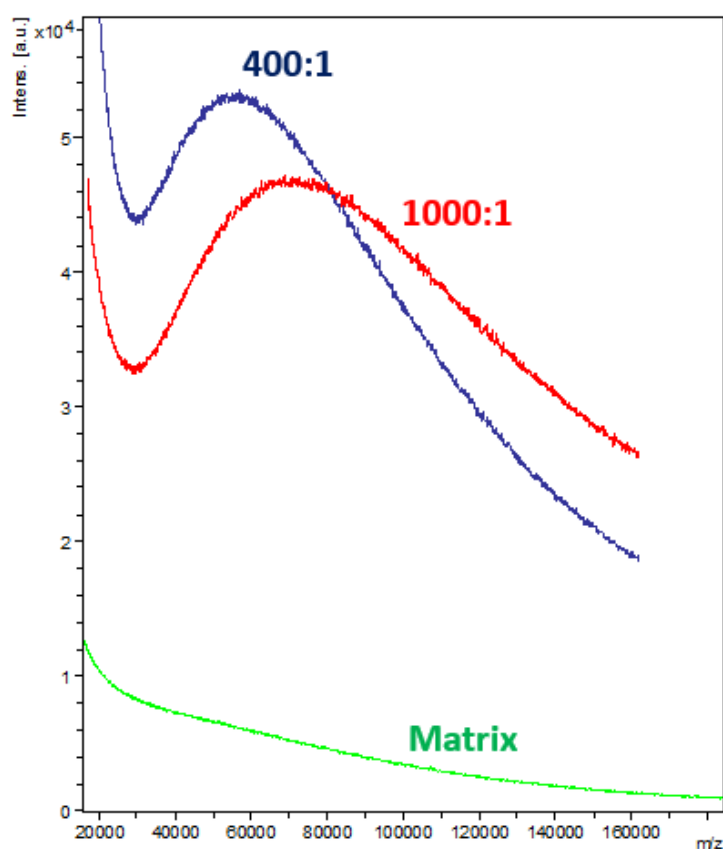


Fig. 2.20. MALDI-MS spectra for high molecular weight polymers; 400:1 (40 kDa, UV-Vis) and 1000:1 (89 kDa, UV-Vis). Linear positive ion mode with mass suppression at 30 kDa and medium signal smoothing.

Although MALDI-MS has been used extensively to provide molecular weight and structural and compositional information of synthetic polymers, it fails to provide accurate molecular weight values for high molecular weight polymers.⁶ However, for the short chain polymer, the

resolution of the mass spectrum was sufficient to characterize average molecular weight, polydispersity and end group analysis.

Average molecular weight determined by using ^1H -NMR, UV-Vis and MALDI-MS for different chain length RAFT-PNIPMAM synthesized by using different proportion of the monomer:CTA ratio are displayed in table 2.6. Average molecular weight values were in reasonably good agreement with the theoretical values. For our purposes, only a crude estimate of M_w is needed for polymer comparison and hence these results were considered adequate.

Table 2.6. Different chain length PNIPMAM synthesized via RAFT polymerization.

Monomer:CTA	Yield (%)	Theoretical molecular weight (kDa)	Average molecular weight (kDa)		
			^1H -NMR	MALDI	UV-Vis
100:1	70	~ 12.7	~ 8.5	~ 5.9	~ 7.5
200:1	68	~ 25.4	~ 20	--	~ 19
300:1	72	~ 38	~ 28	--	~ 27
400:1	80	~ 50	~ 43	--	~ 40
1000:1	76	~ 127	--	--	~ 89

2.4.3.2. Molecular weight characterization of ATRP-PNIPMAM using MALDI-MS

For short chain polymers synthesized via ATRP, the reason for getting lower molecular mass polymer was not clear. ATRP of acrylamide is known to have problems such as deactivation of the catalyst and early termination due to removal of terminal bromine. Therefore, we tried to characterize ATRP polymers end groups using MALDI-MS to find any possible reason behind the unsuccessful polymerization.

For PNIPMAM synthesized via ATRP by direct monomer polymerization, a different matrix 2,5-dihydroxybenzoic acid (DHB) and similar spotting condition (premix followed by spotting) was used for the sample preparation. The M_w of the polymer was 4270.6 Da with a PDI of 1.02 (Fig. 2.21).

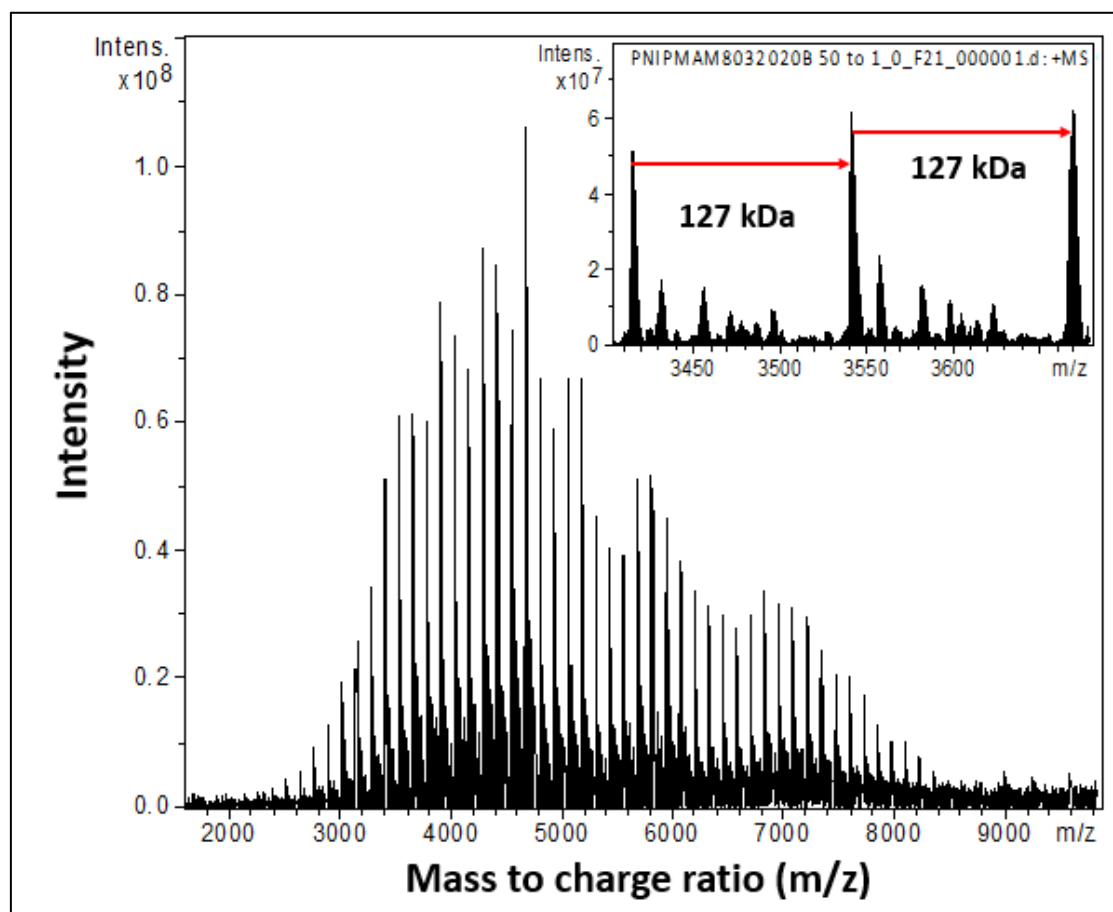


Fig. 2.21. MALDI-MS of ATRP-PNIPMAM ($M_n = 4174.8$ Da, $M_w = 4270.64$ Da and Poly-dispersity index ($M_w/M_n = 1.02$). Distance between repeat units = 127 Da (inset).

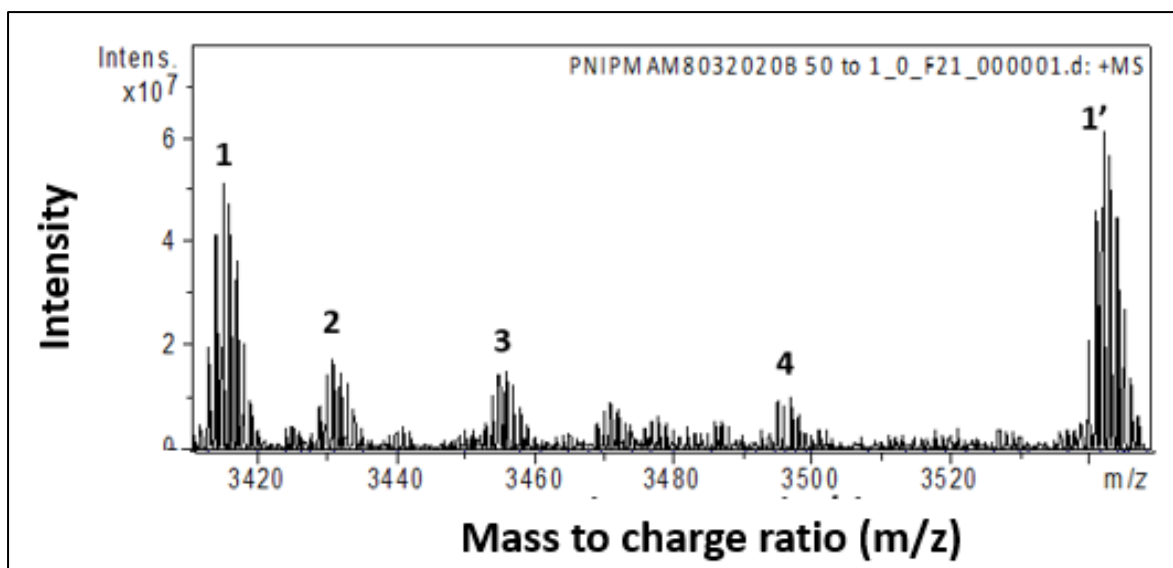
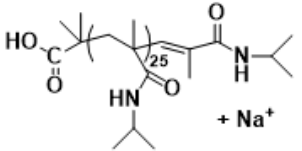
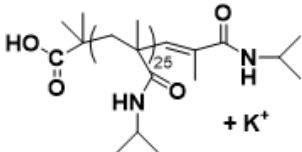
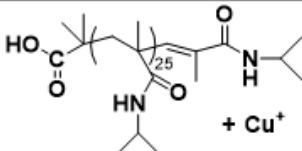
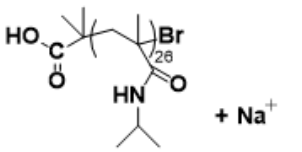


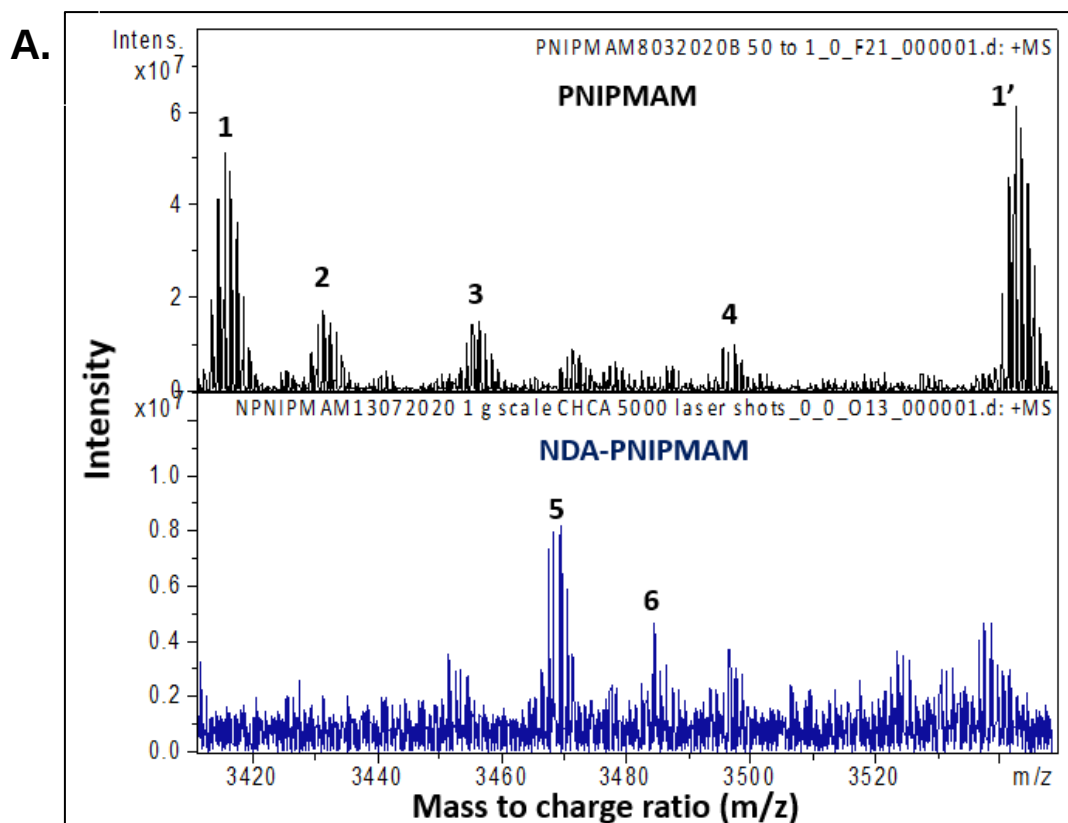
Fig. 2.22. End group analysis of ATRP-PNIPMAM: A set of four peaks obtained in the MALDI-MS of the polymer. Molecular formulas and predicted structures were given in table 2.7.

The end groups were identified by analysing the isotopic distribution pattern and corresponding mass for the major polymer peak. The expanding spectrum revealed a repeating set of four peaks which were separated from the neighbouring sets by monomer molecular weight (127 Da), which confirmed the successful synthesis of PNIPMAM (**Fig. 2.22**). The four peaks were attributed to the chains bearing an isobutyric acid ($C_4H_7O_2$) residue at one end and alkene or bromo residues at the other end. For instance, the major alkene terminated peak at $m/z = 3415.6$ corresponds to $C_4H_7O_2(C_7H_{13}ON)_{25}C_7H_{12}ON/Na^+$ and the bromine terminated peak at $m/z = 3496.5$ corresponds to $C_4H_7O_2(C_7H_{13}ON)_{26}Br/Na^+$ (**Table 2. 7**).

Table 2.7. Molecular formulas and predicted structures corresponding to the peaks obtained in the MALDI-MS of ATRP-PNIPMAM (**Fig. 2.22**). Major peak at $m/z = 3415.6$ was corresponded to an alkene terminated molecular ion $C_4H_7O_2(C_7H_{13}ON)_{25}C_7H_{12}ONNa^+$.

Peak No.	Molecular formula	m/z	Predicted structure
1	$C_4H_7O_2(C_7H_{13}ON)_{25}C_7H_{12}ONNa^+$	3415.6	
2	$C_4H_7O_2(C_7H_{13}ON)_{25}C_7H_{12}ONK^+$	3431.6	
3	$C_4H_7O_2(C_7H_{13}ON)_{25}C_7H_{12}ONCu^+$	3456.6	
4	$C_4H_7O_2(C_7H_{13}ON)_{26}BrNa^+$	3496.5	

Presence of bromine-terminated peak pointed towards a successful ATRP synthesis but the origin of the major alkene terminated peak was unclear. Alkene terminated peaks formed during polymers ionization in MALDI-MS were reported by several researchers.^{2,44} However, this mixture of alkene and bromine terminated peaks could also be due to PNIPMAM having a mixture of end groups. It could form due to partial termination during the polymerization (removal of bromine).



B.

Peak No.	Molecular formula	m/z	Predicted structure
5	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}C_7H_{12}ONNa^+$	3468.5	
6	$C_{12}H_{15}O_5N_2(C_7H_{13}ON)_{24}C_7H_{12}ONK^+$	3484.5	

Fig. 2.23. A.) MALDI-MS spectra of PNIPMAM and NDA-PNIPMAM showing the complete disappearance of the major alkene terminated peak of PNIPMAM and appearance of new peaks in NDA-PNIPMAM. B) A table containing molecular formulas and predicted structures corresponding to the peaks obtained in the MALDI-MS of NDA-PNIPMAM. The structure of the major peak corresponding to ion $C_{12}H_{15}N_2O_5(C_7H_{13}ON)_{24}C_7H_{12}ONNa^+$ with the calculated mass 3468.5 Da.

MALDI-MS also confirmed a complete conversion of PNIPMAM to NDA-PNIPMAM (**Fig. 2.23**). The major alkene terminated peak in PNIPMAM was completely replaced by a new peak, which has a mass corresponding to NDA coupled to the end group of the alkene-terminated peak of PNIPMAM (**Fig. 2.23 (B)**). In conclusion, characterisation of M_w with this method is not great and limited to small M_w ; however, we confirmed chemical structure including end-group functionalities.

2.4.3.3. Molecular weight characterization of ATRP-PMASI using MALDI-MS

For PMASI, DHB was a suitable matrix and the m/z distribution was found to be between 700 – 5000 Da, with M_w of 3172.5 Da and PDI of 1.12 (**Fig. 2.24**). The peak spacing between polymer units was ~ 183 Da apart, correlating with the mass of monomeric units of the PMASI. The end group analysis of PMASI revealed the presence of a set of 4 peaks, where, the major peak was attributed to have an alkene end group ($m/z = 1968.6$, **Table. 2.8**). The reason for obtaining low molecular weight PMASI could be an early termination of polymerization caused by the removal of HBr leading to the unsaturation in the polymer. As explained earlier, it could also happen during the polymer ionization in MALDI. Therefore, it is difficult to comment on the reason for not getting expected molecular weight PMASI using ATRP.

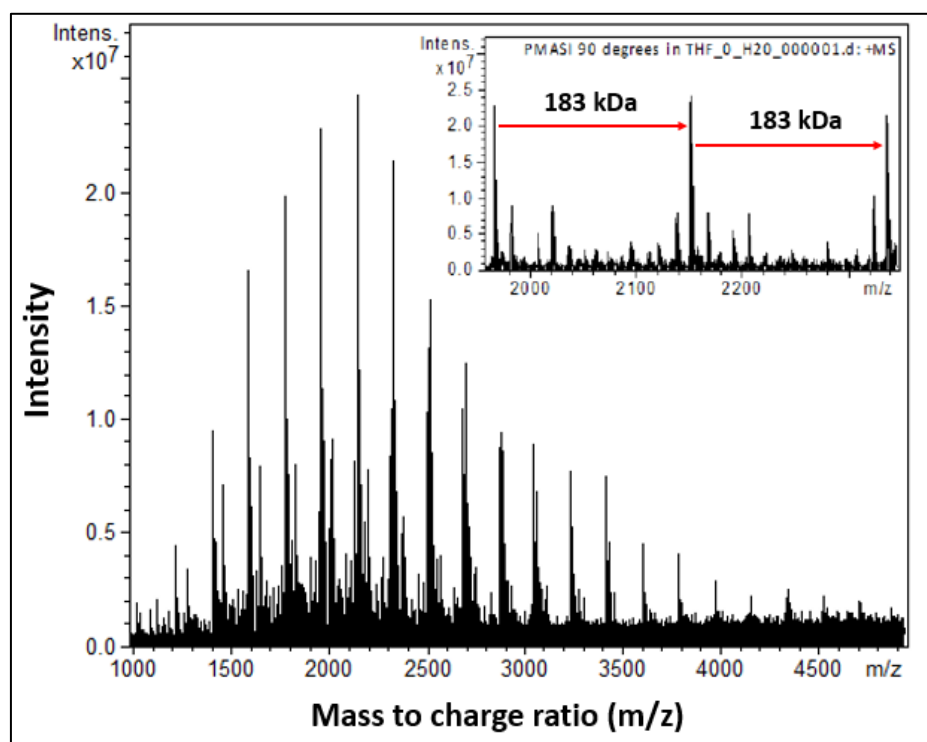


Fig. 2.24. MALDI-MS spectrum (MALDI-MS) of PMASI: Spectra from 1 mg/mL PMASI at dilutions of 1/10 prepared in a 20 mg/mL matrix solution of dihydroxybenzoic acid (DHB) in MeCN was acquired between mass range 1000 - 5000 Da, inlet (top right) shows spacing between polymer peaks of 183 Da. $M_n = 2759.9$ Da, $M_w = 3172.5$ Da, PDI = 1.14.

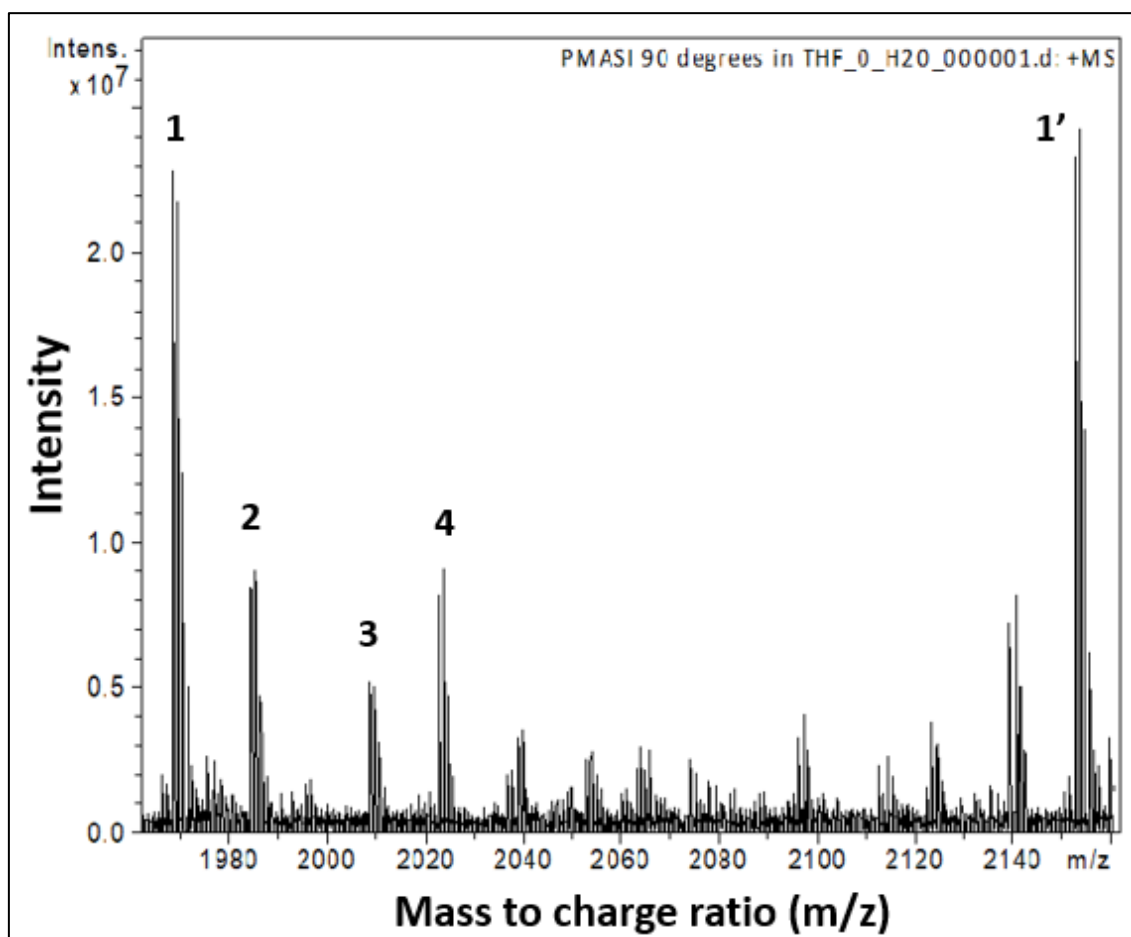
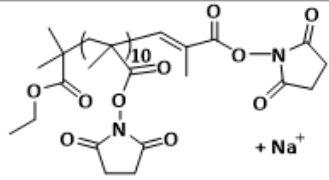
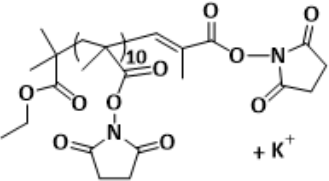
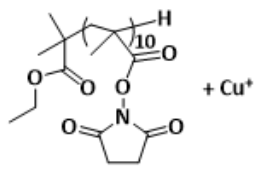
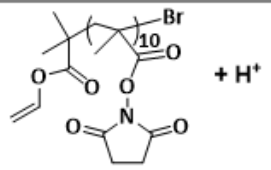


Fig. 2.25. End group analysis of PMASI: A set of four peaks obtained in the MALDI-MS of the polymer. Molecular formulas and predicted structures were given in the table 2.8.

Table 2.8. Molecular formulas and predicted structures corresponding to the peaks obtained in the MALDI-MS of PMASI (**Fig. 2.25**). The structure of the major peak corresponding to ion $C_6H_{11}O_2(C_8H_9O_4N)_9C_8H_8O_4NNa^+$ with the calculated mass 1968.6 Da.

Peak No.	Molecular formula	m/z	Predicted structure
1	$C_6H_{11}O_2(C_8H_9O_4N)_9C_8H_8O_4NNa^+$	1968.6	 + Na ⁺
2	$C_6H_{11}O_2(C_8H_9O_4N)_9C_8H_8O_4NK^+$	1984.6	 + K ⁺
3	$C_6H_{11}O_2(C_8H_9O_4N)_{10}HCu^+$	2011.5	 + Cu ⁺
4	$C_6H_9O_2(C_8H_9O_4N)_{10}BrH^+$	2025.5	 + H ⁺

After the successful characterization of all chain length PNIPMAM (RAFT and ATRP), the next step was to check their phase transition properties, which are described in the next section.

2.4.4. Phase transition studies on PNIPMAM

The reported methods in the literature to study the LCST are differential scanning calorimetry (DSC) and UV-Vis spectrophotometry.^{2,16-17} DSC detects the heat change whereas UV detects the light scattering caused by the precipitation of polymer. DSC instrument available in the department (DSC822e differential scanning calorimeter) did not have sufficient sensitivity to detect the phase transition of PNIPMAM. During phase transition studies using UV-Vis, the data obtained was unreliable. The main difficulty was the efficient

temperature control during UV measurements. Since UV-Vis is a large-scale turbidimetry method, sample transparency was also an issue. After the on-set of phase transition, the solution became inhomogeneous. The bigger precipitates were settling down and the transmittance was randomly fluctuating due to arbitrary light scattering by different sized precipitates. Hence nano-differential scanning fluorimetry (NanoDSF) was used as a small-scale turbidimetry method with excellent temperature control. NanoDSF is normally used to check the protein stability with respect to change in temperature. It also allows detecting the intensity of scattered light (at 350 nm) with the change in temperature and hence we found it was well suited for studying PNIPMAM phase transition. During the optimization of the measurement conditions for Nano-DSF, we observed a concentration dependence on the phase transition. The LCST was observed to increase with decrease in the weight concentration of the polymer sample (**Fig. 2.26**). There are several reports in literature supporting our results, where researchers observed a strong dependence of concentration on the phase transition of PNIPAM.⁴⁷⁻⁴⁸ Boutris *et. al.* observed an increase in phase transition temperature from 32 °C to 42 °C when decreased the concentration from 15 wt% to 2 wt% PNIPAM.⁴⁷

In the literature, the characteristic phase separation temperatures were reported either as the onset of the transition⁴⁹ or as the temperature corresponding to the peak rate of scattering intensity change.⁵⁰ For our studies, the latter temperatures were reported as polymer LCST (**Table 2.9**). For different chain length polymers, we observed an increase in the LCST with decrease in PNIPMAM molecular weight (**Fig. 2.27, 2.28**). This molecular weight dependence on the polymer LCST is also reported in the literature.¹⁶

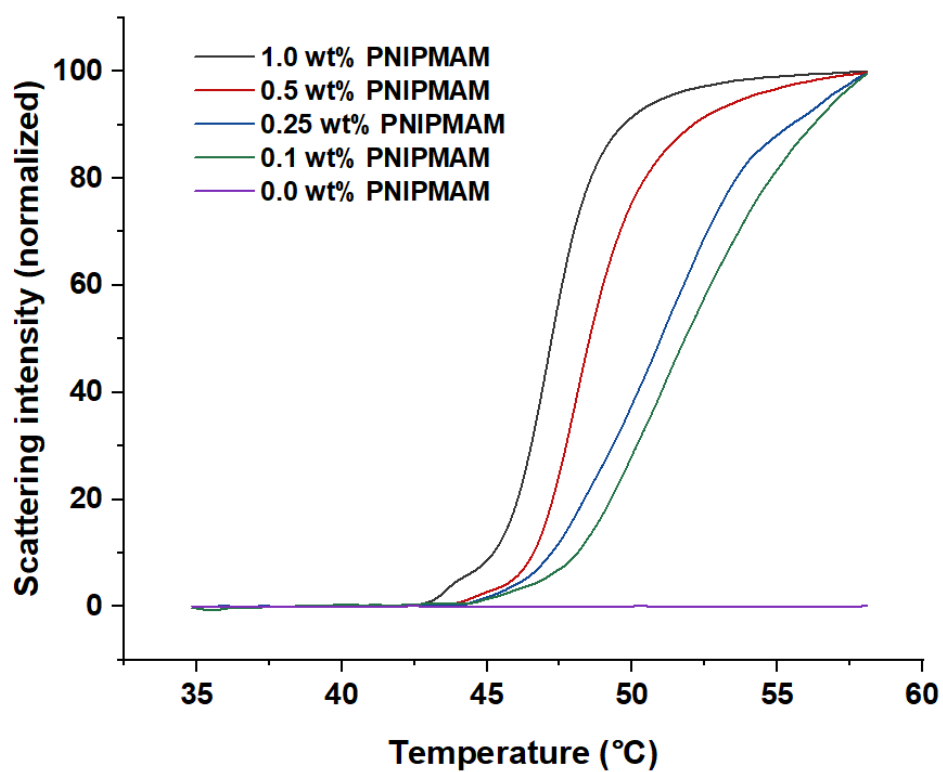


Fig. 2.26. Scattering intensity vs. temperature plot to show concentration dependance of PNIPMAM (400:1, 40 kDa) on its LCST.

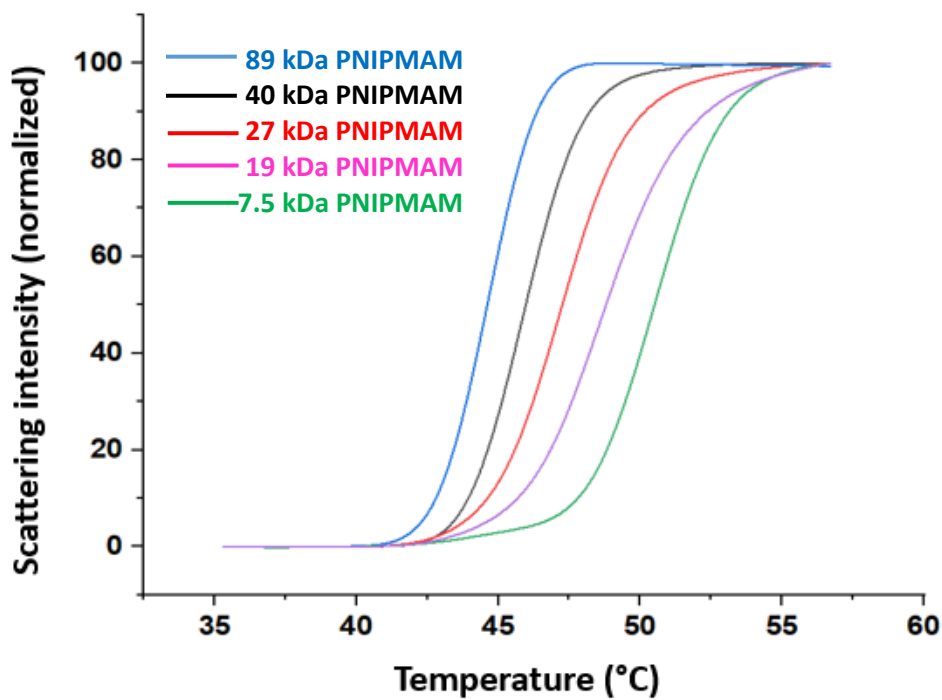


Fig. 2.27. PNIPMAM phase transition study using NanoDSF: Scattering intensity vs. temperature plot of different chain length RAFT-PNIPMAM (0.5 mM).

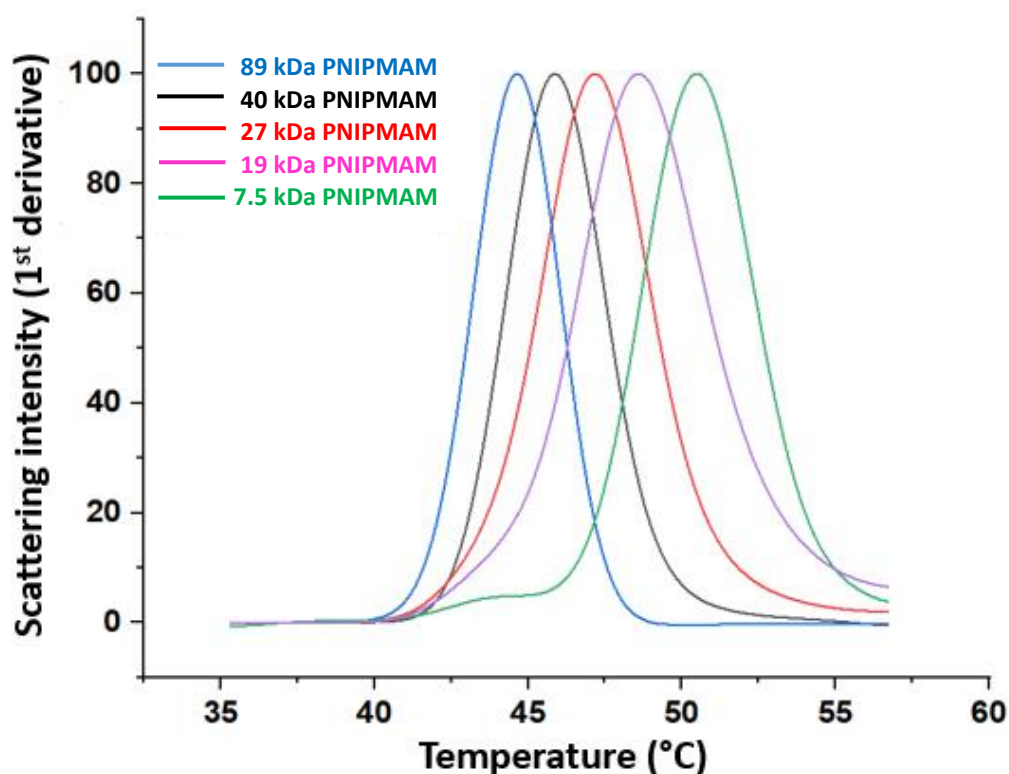


Fig. 2.28. PNIPMAM phase transition study using NanoDSF: Plot of scattering intensity (first derivative) with temperature showing the peak temperature of thermogram (0.5 mM).

For PNIPMAM synthesized via ATRP (~ 4 kDa), both PNIPMAM and NDA-PNIPMAM were found to have LCST above 40 °C (**Fig. 2.29**). There was a drop in phase transition temperature of PNIPMAM (~ 50 °C) after NDA functionalization (~ 45 °C). This drop in the LCST was likely due to the purification of NDA-PNIPMAM where, the polymer fraction with higher phase transition was lost during the washings.

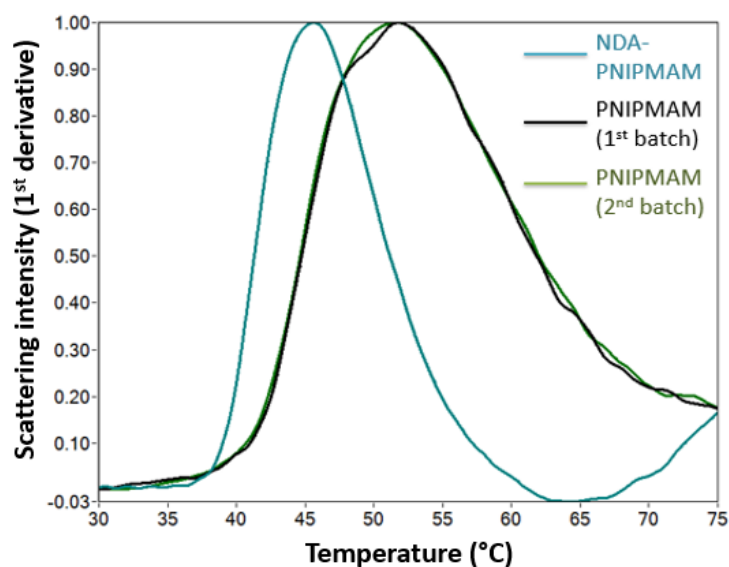


Fig. 2.29. Plot of scattering intensity (first derivative) with temperature to calculate LCST of NDA-PNIPMAM and two different batches of PNIPMAM polymer (0.5 mM).

The NanoDSF results for different chain length PNIPMAM are summarized in the **table 2.9** where a decrease in the LCST was observed with increase in the molecular weight of PNIPMAM.

Table 2.9. Dependence of LCST on the molecular weight of PNIPMAM (0.5 mM).

PNIPMAM (kDa)	LCST (°C)
4*	51.7
7.5	50.5
19	48.6
27	47.2
40	45.9
89	44.6

Note. * ATRP-PNIPMAM ~ 4 kDa (MALDI-MS)

2.5. Conclusions

In this chapter, a range of PNIPMAM polymers with tuneable molecular weight (from 7.5 kDa to 89 kDa) was prepared using RAFT polymerization method. The polymers were end-functionalized with NDA. The structure of the polymer was confirmed using ^1H -NMR and molecular weight distribution was analysed using UV-Vis spectroscopy and MALDI-MS. Even though we could not use GPC, a combination of these alternative methods (^1H -NMR, UV-Vis and MALDI-MS) gave consistent estimate of polymer molecular weight. End group analysis of both short chain ATRP and RAFT PNIPMAM was done using MALDI-MS. Conversion of PNIPMAM to NDA-PNIPMAM was also monitored using MALDI-MS. The phase transition behaviour of different chain length polymers was characterized using Nano-DSF, which measures change in the scattering intensity with respect to change in temperature. The results showed a concentration dependence on PNIPMAM phase transition where the LCST increased with decrease in the weight percentage of polymer. The phase transition was also dependent on the molecular weight of PNIPMAM where the LCST decreased with increase in the chain length of the polymer. Similar studies are reported on RAFT and ATRP synthesis of different M_w PNIPMAM and their phase transition behaviour.^{16,25} Hence, the novelty of this work is associated with the methodology, specifically with the NanoDSF. Light scattering outcomes of the instrument were explored as a potential method for the phase transition studies of PNIPMAM. It could further be explored as a sensitive tool for studying such properties of other thermo-responsive polymers.

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Chapter 3: IONPs synthesis

3.1. Introduction

Particle size and magnetic heating are two important parameters which determine the potential of functional IONPs for the biomedical applications.¹⁻⁴ Specific absorption rate (SAR) of IONPs depends on NPs size and the magnetic parameters for the measurement.¹⁻⁴ Hence, one objective was to tune NP size to achieve optimum SAR. Apart from particle size, the heating efficiency of magnetic NPs is strongly dependent on their magnetic anisotropy which is a morphology effect.^{4,14} Due to low magnetic anisotropy as compared to their spherical counterparts, cubic shaped IONPs could also be a desirable candidate for the protein delivery applications.¹⁴

In a preliminary study, 6 nm IONPs coated with thermally responsive poly-*N*-isopropylacrylamide (PNIPAM) showed a potential for protein delivery applications, but, their magnetic heating was low (SAR = 3.6 W/g).¹¹ Therefore, to have a better understanding of magnetic heating behaviour of IONPs, different size and shape IONPs have been synthesized and studied. This chapter will first describe synthesis and characterisation of IONPs using thermal decomposition and polyol methods, followed by their magnetic characterization (SAR values).

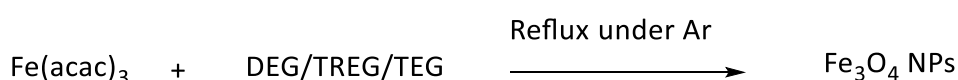
3.1.1. Synthesis of various size and shaped IONPs

3.1.1.1. Polyol synthesis

Water soluble 6 nm IONPs were previously synthesized using a modified polyol synthesis, by the precipitation from FeCl₃ and NaOH in diethylene glycol (DEG).¹¹ Here, Fe(III) is partially reduced to Fe(II) which then nucleate to form mixed metal oxide IONPs at high temperature (180 °C). Several reports investigated and demonstrated the effect of chain length on the properties of the nanoparticles. Hachani *et. al.* synthesized IONPs using different chain length glycols such as DEG, triethylene glycol (TREG) and tetra-ethylene glycol (TEG).⁹ They reported

a correlation between the size of glycol and size of NPs, as the greater the length of the glycol, the larger the size of the synthesized NPs. However, some reports suggested poor stability of TREG and TEG coated IONPs which could be resolved by ligand exchange with better stabilising ligands.⁹ In this section, the effect of polyol chain length (TREG and TEG) on NPs size and shape has been studied.

Different size magnetic IONPs were synthesized using a modified polyol synthesis procedure⁷ by the thermal decomposition of $\text{Fe}(\text{acac})_3$ in different polyols (**Scheme 3.1**).



Scheme 3.1. Synthesis of IONPs in various polyol solvents.

The successful Fe_3O_4 NPs synthesis of TREG @ 8.4 ± 2.2 nm IONPs was confirmed by powder X-ray diffraction (XRD) where, the characteristic diffraction peaks at 30.1° , 35.4° , 43.1° , 53.4° , 57.0° and 62.6° were indexed to the planes (220), (311), (400), (422), (511) and (440) of magnetite (JCPDS 87-0245)¹⁷, respectively (**Fig. 3.1 (A)**).

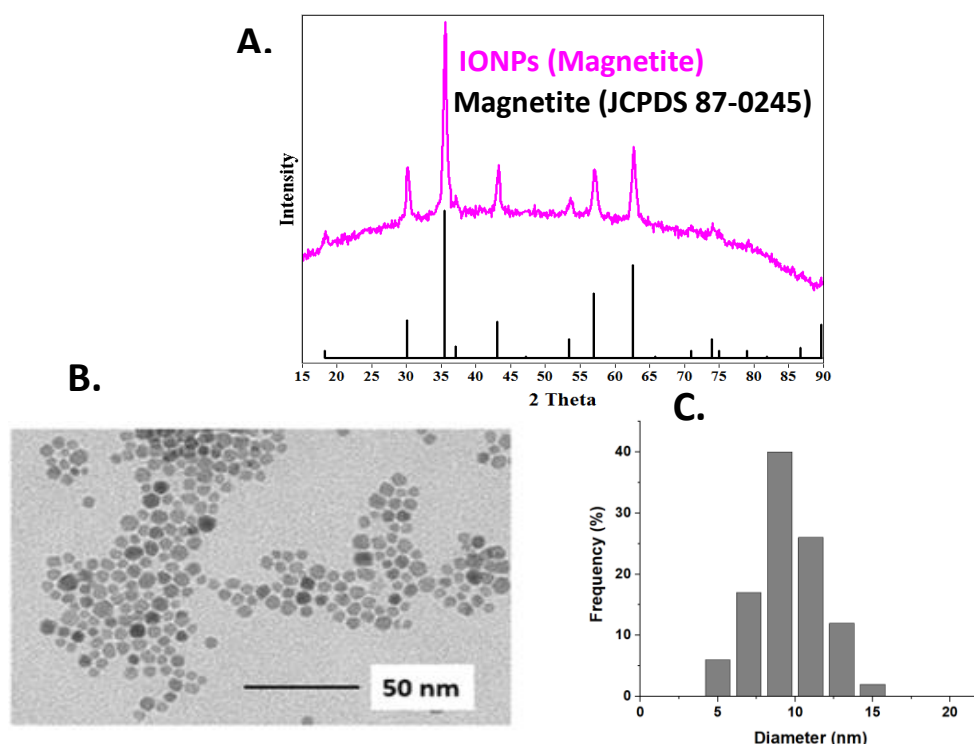


Fig. 3.1. Polyol synthesis in TREG: (A) XRD pattern, (B) TEM image and (C) size distribution histogram analysed by TEM of 8.4 ± 2.2 nm IONPs ($\pm$ denoted standard deviation, $n = 103$).

Transmission electron microscopy (TEM) was used to monitor the NPs size where, size distribution histogram analysis of TEM images revealed the average diameter of IONPs. Various size IONPs were obtained with different chain length polyol: DEG (6 ± 1 nm), TREG (8.4 ± 2.2 nm) and TEG (10.1 ± 2.6 nm). With DEG and TREG, IONPs were forming a stable suspension (in water) but, with TEG, the NPs were quickly precipitating due to agglomeration (Fig. 3.2).

Table 3.1. Conditions for the synthesis of different size IONPs in various polyols

Solvent	Time (hr)	Reflux temperature (°C)	Average diameter (nm)
DEG	2	180	6 ± 1
TREG	2	280	8.4 ± 2.2
TEG	2	310	10.1 ± 2.6
TREG	4	280	11.3 ± 2.3

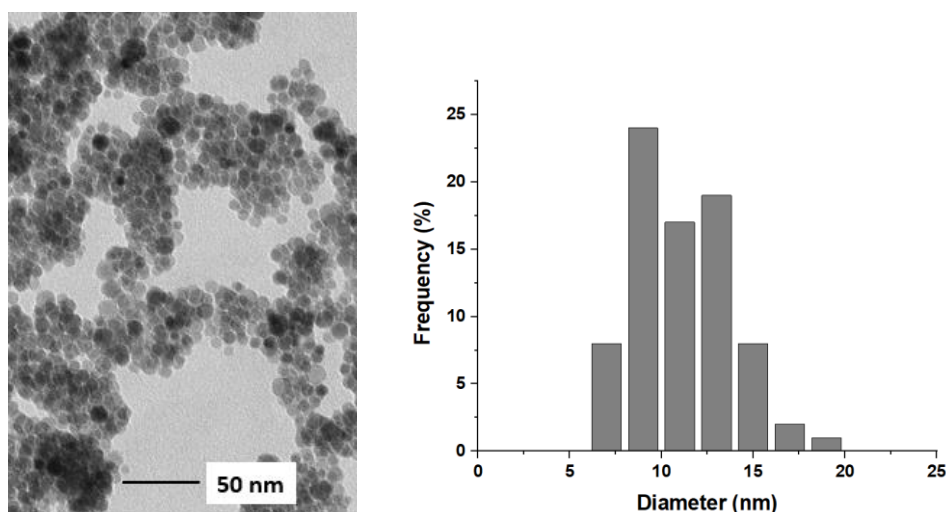


Fig. 3.2. Polyol synthesis in TEG: TEM image and size distribution histogram analysed by TEM of 11.3 ± 2.3 nm IONPs ($\pm$ denoted standard deviation, $n = 79$).

Since TREG yielded stable IONPs, we optimized other synthesis parameters to increase the NPs size further with TREG. Longer reflux time leads to bigger NPs due to increased Ostwald ripening and coalescence.¹⁵⁻¹⁶ We managed to increase NPs diameter in TREG from 8.4 ± 2.2 nm to 11.3 ± 2.3 nm by increasing the reflux time from 2 h to 4 h (**Fig. 3.3**). However, 11.3 ± 2.3 nm IONPs did not form a stable suspension in water possibly due to agglomeration.

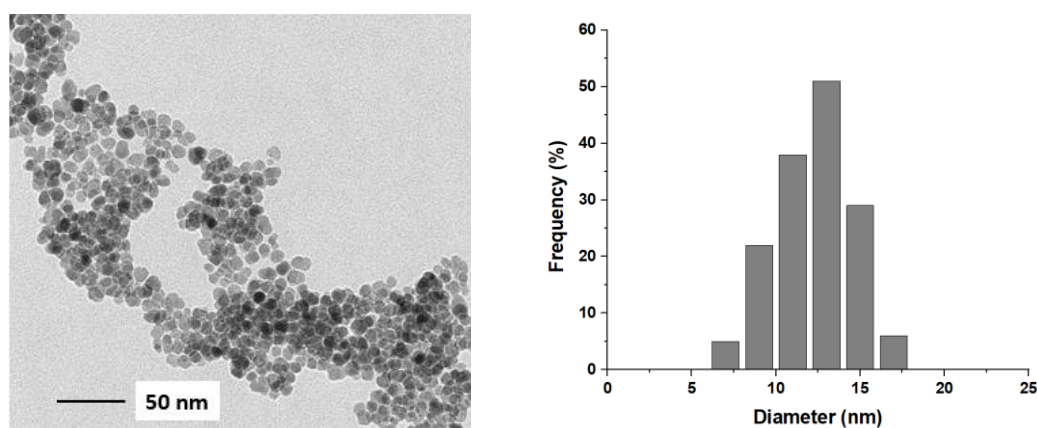


Fig. 3.3. Polyol synthesis in TREG: TEM image and size distribution histogram analysed by TEM of 11.3 ± 2.3 nm IONPs ($\pm$ denoted standard deviation, $n = 151$).

Increase in NPs diameter using polyol synthesis resulted in a considerable increase of the agglomeration. Therefore, it was decided to use a different approach to obtain stable IONPs. Thermal decomposition of $(\text{Fe}(\text{acac}))_3$ in the presence of oleic acid and oleyl amine is reported as a procedure to synthesize IONPs with well-defined shapes and sizes.¹ Similar to the polyol, the ligands in thermal decomposition are used as stabilisers chemisorbed on the surface of the magnetic NPs. However, unlike polyol, they assist the NPs dispersion in organic solvents and require ligand exchange to make them water soluble.

In the next section, synthesis and characterization of different size and shape IONPs using thermal decomposition of $\text{Fe}(\text{acac})_3$ has been reported. Subsequently, a successful ligand exchange strategy to obtain water dispersible PNIPMAM@IONPs has been discussed.

3.1.1.2. Thermal decomposition of Fe(acac)₃: Synthesis of different size IONPs

IONPs with average diameter 11 nm and 16 nm were obtained via a solvent free thermal decomposition/reduction of Fe(acac)₃ in oleylamine/oleic acid (**Fig. 3.4**).

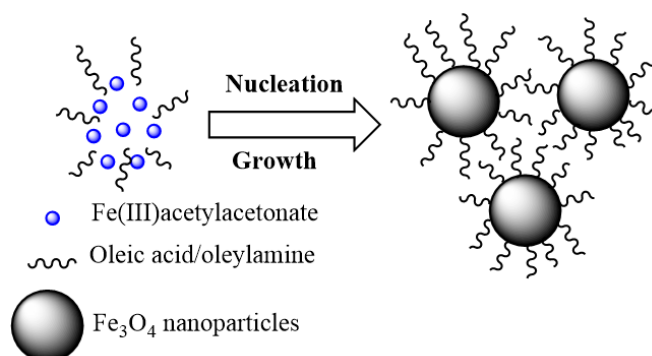
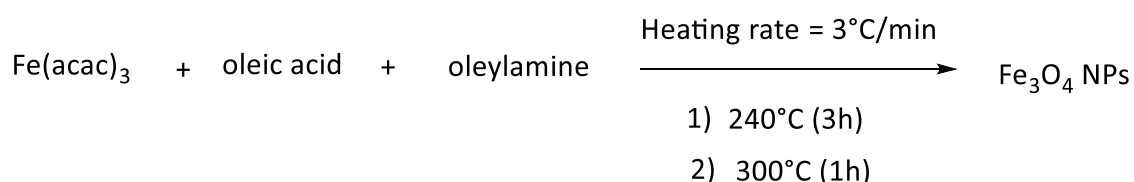


Fig. 3.4. Synthesis of oleic acid/oleylamine coated IONPs using thermal decomposition of Fe(acac)₃.

Synthesis of 16 nm IONPs was adapted from a previous study by Mohapatra *et. al.* with some modifications (**Scheme 3.2**).¹



Scheme 3.2. Synthesis of 16 nm spherical IONPs by thermal decomposition of Fe(acac)₃. Optimized conditions: mole ratio = Fe(acac)₃ : oleylamine : oleic acid = 1:2:1, heating rate = 3°C/min under argon.

The successful Fe₃O₄ NPs synthesis was confirmed by powder X-ray diffraction (XRD). The characteristic diffraction peaks in XRD pattern at 30.1°, 35.4°, 43.1°, 53.4°, 57.0° and 62.6° were indexed to the planes (220), (311), (400), (422), (511) and (440) of magnetite (JCPDS 87-0245)¹⁷ respectively (**Fig. 3.5 (A)**). Selected area diffraction (SAED) pattern (**Fig. 3.5 (E)**) showed

discrete spots indexed to the (111), (220), (311), (400), (422), (511) and (440) planes of magnetite. The transmission electron microscopy (TEM) images (**Fig. 3.5 (B)**) and size distribution histogram analysis of TEM (**Fig. 3.5 (C)**) demonstrated the average particle diameter to be 15.4 ± 2.1 nm ($\pm$ denoted standard deviation, $n = 111$). Furthermore, the high-resolution TEM image (**Fig. 3.5 (B)**) taken from an individual particle reveals well-resolved lattice fringes with an inter-planar distance of 0.51 nm and 0.32 nm indexed to the (111) and (220) planes of magnetite respectively (**Fig. 3.5 (D)**).

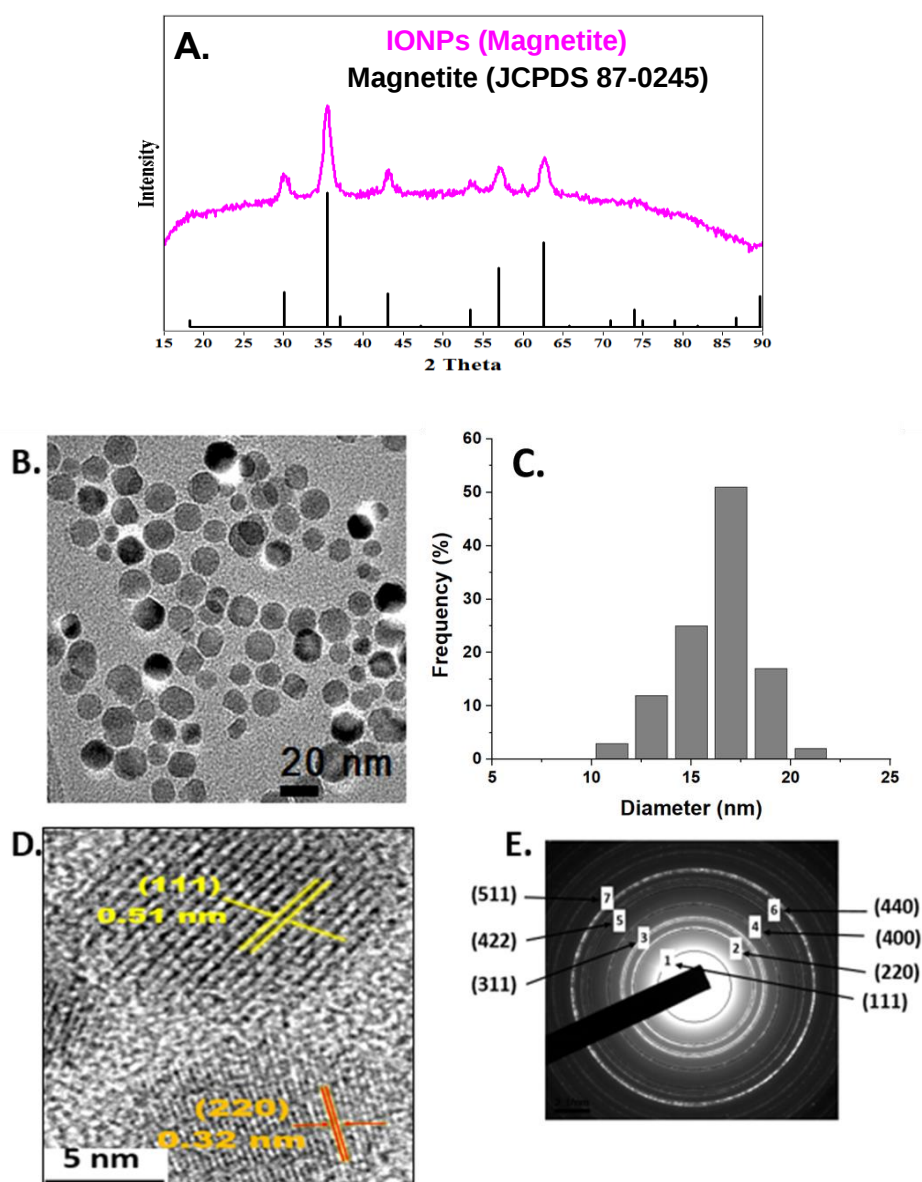


Fig. 3.5. Synthesis of 16 nm spherical IONPs: (A) XRD pattern, (B) TEM image, (C) size distribution histogram analysed by TEM, (D) HRTEM image and (E) SAED pattern taken from 15.4 ± 2.1 nm IONPs ($\pm$ denoted standard deviation, $n = 111$). Optimized conditions: mole ratio = $\text{Fe}(\text{acac})_3$: oleylamine : oleic acid = 1:2:1, heating rate = 3 °C/min under argon.

Mohapatra *et. al.* observed a decrease in the NPs size with increase in the oleylamine concentration during synthesis.¹ It could be due to chemisorption of oleylamine molecules on NPs surface restricting the particle growth and ripening process. We also observed a decrease in IONPs size with increase in the oleylamine concentration from 16 nm to 11nm (**Fig. 3.6**).

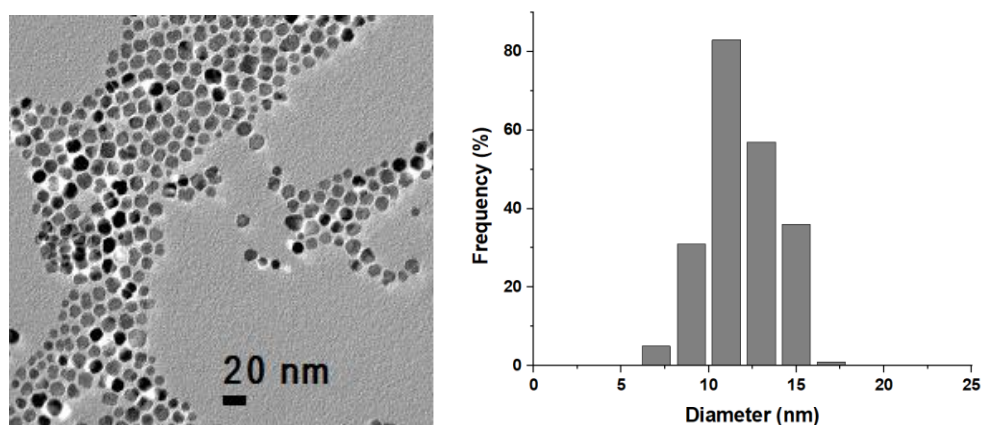


Fig. 3.6. Synthesis of 11 nm spherical IONPs: TEM image and size distribution histogram analysed by TEM of 11 ± 2 nm IONPs ($\pm$ denoted standard deviation, $n = 133$). Optimized conditions: mole ratio = $\text{Fe}(\text{acac})_3$: oleylamine : oleic acid = 1:3:1, heating rate = 5°C/min under argon.

We tried to increase NPs size (> 16 nm) by changing the molar ratio of acid to amine in the synthesis. There was a sudden increase in NPs size from 16 nm to 100 nm when similar concentration of oleic acid and oleylamine was used in the synthesis (**Fig. 3.7**). This size increase with increase in oleic acid concentration was in accordance with the literature.^{1,16} More optimization was required (reflux time and temperature, different molar ratio) to synthesize a range of different size IONPs. However, because of the time constraint, we decided to use other approaches to prepare bigger NPs.

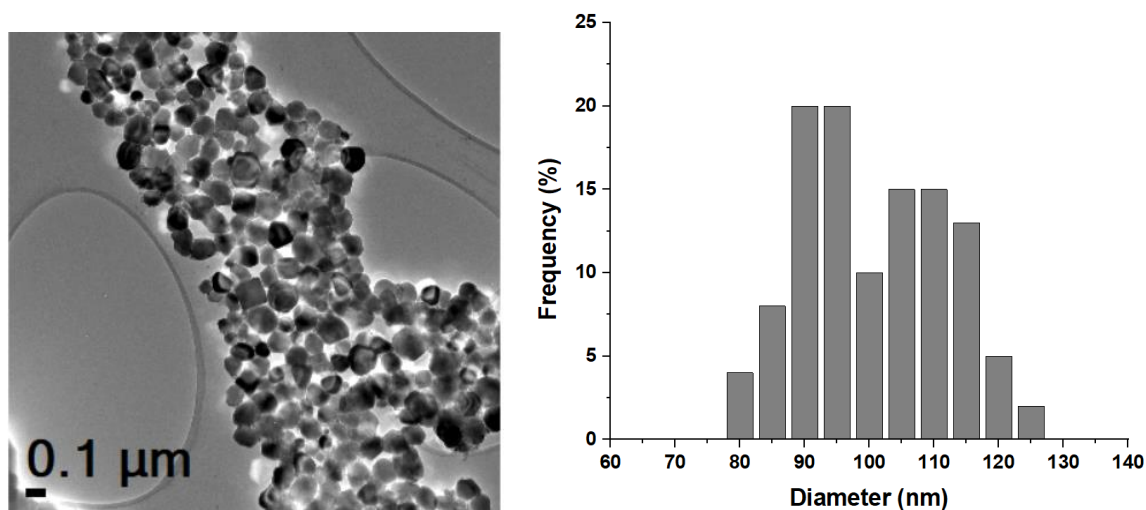
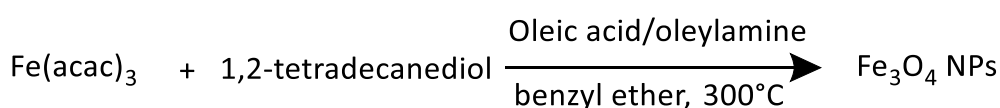


Fig. 3.7. Synthesis of 100 nm spherical IONPs: TEM image and size distribution histogram analysed by TEM of 100.3 ± 11.4 nm IONPs ($\pm$ denoted standard deviation, $n = 116$). Optimized conditions: mole ratio = $\text{Fe}(\text{acac})_3$: oleylamine : oleic acid = 1:3:3, heating rate = $3^\circ\text{C}/\text{min}$ under argon ($240^\circ\text{C} = 1\text{h}$, $300^\circ\text{C} = 3\text{h}$).

Tong *et. al.* reported a successful seed mediated approach to synthesize different size IONPs (6 - 40 nm) with controlled dispersity.² Hence, it was decided to use a similar approach for preparing bigger IONPs (> 16 nm). Seeds were synthesized by refluxing a mixture of $\text{Fe}(\text{acac})_3$, 1,2-tetradecanediol, oleic acid and oleylamine in benzyl ether at 300°C (**Scheme 3.3**).



Scheme 3.3. Synthesis of 7 nm faceted IONPs by thermal decomposition of $\text{Fe}(\text{acac})_3$. Optimized conditions: mole ratio = $\text{Fe}(\text{acac})_3$: 1,2-tetradecanediol : oleylamine : oleic acid = 1:5:6:6, 20 ml benzyl ether, heating rate = $5^\circ\text{C}/\text{min}$ under argon.

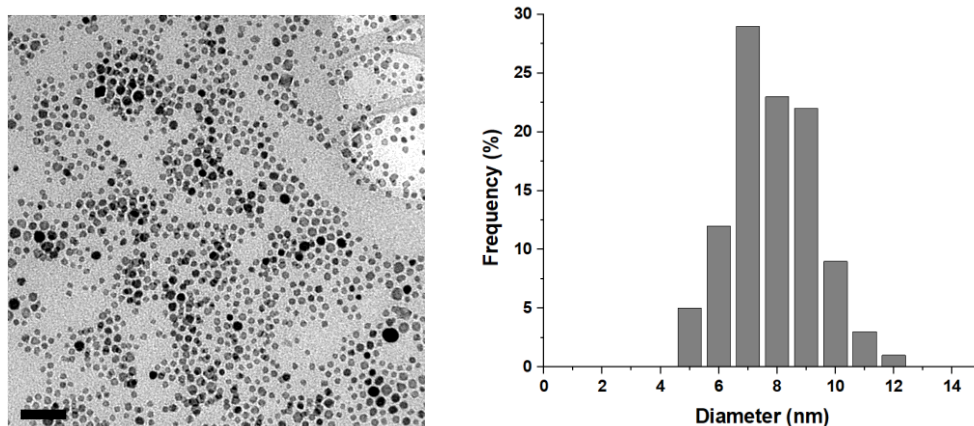


Fig. 3.8. Synthesis of 7 nm cubic IONPs: TEM image and size distribution histogram analysed by TEM of 7.3 ± 1.4 nm IONPs ($\pm$ denoted standard deviation, $n = 104$).

It was possible to increase the particle size with this seed mediated approach but, the size distribution (**Fig. 3.9**) was broad (9 ± 6 nm). Extensive optimization of seed mediated method was required and hence this method was not suitable for obtaining bigger NPs.

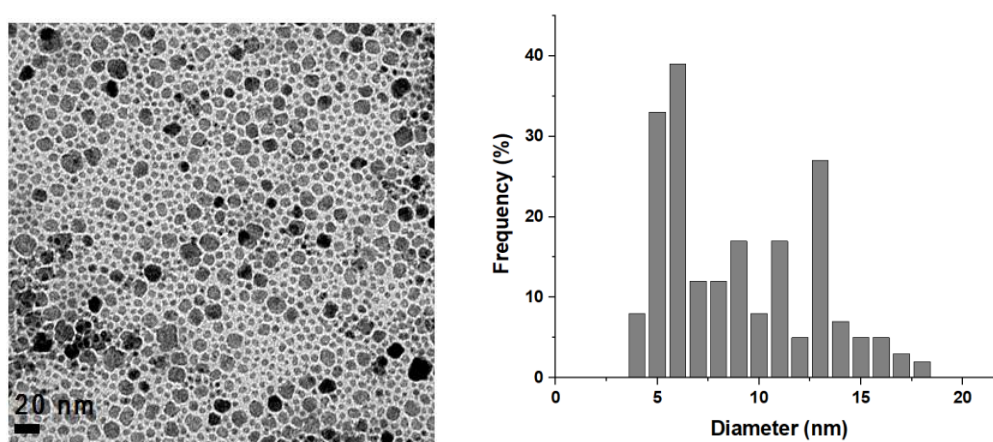
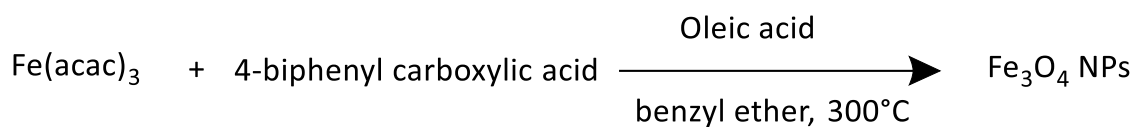


Fig. 3.9. Synthesis of bigger NPs using 7 nm IONPs as seeds: TEM image and size distribution histogram analysed by TEM of 9 ± 6 nm IONPs ($\pm$ denoted standard deviation, $n = 209$).

Kim *et. al.* reported a synthesis approach to obtain 20 to 160 nm IONPs.¹⁶ In a typical synthesis, $\text{Fe}(\text{acac})_3$ with different ratios of 4-biphenylcarboxylic acid and oleic acid in benzyl ether was refluxed at a high temperature (290 °C).



Scheme 3.4. Synthesis of bigger IONPs by thermal decomposition of $\text{Fe}(\text{acac})_3$ in 4-biphenyl carboxylic acid/oleic acid ligand system.

The transmission electron microscopy (TEM) images (**Fig. 3.10**) and size distribution histogram analysis of TEM demonstrated the average particle diameter to be 27.4 ± 3.6 nm ($\pm$ denoted standard deviation, $n = 113$).

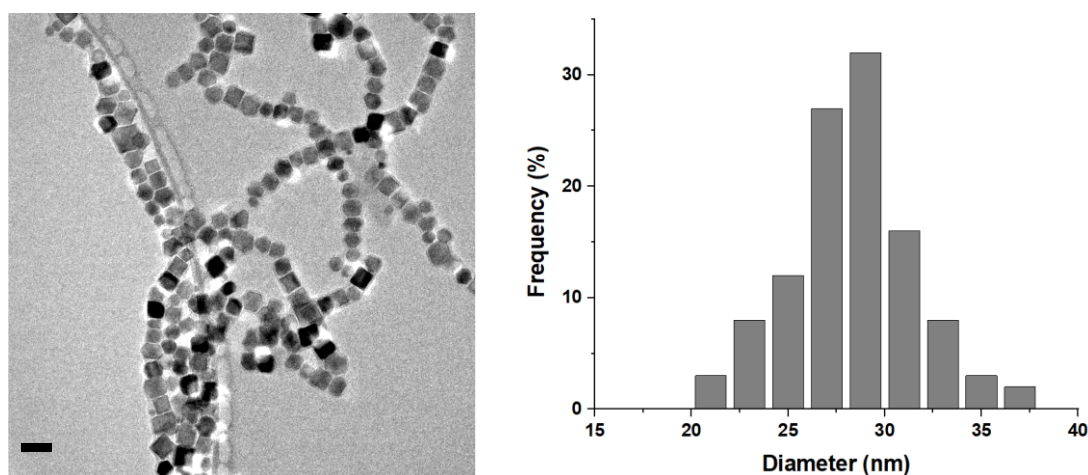


Fig. 3.10. Synthesis of 27 nm cubic IONPs: TEM image and size distribution histogram analysed by TEM of 27.4 ± 3.6 nm IONPs ($\pm$ denoted standard deviation, $n = 113$).

NPs up to 16 nm, irrespective of their shape, were fully dispersed in the reaction mixture without any attraction to the magnetic bead. With the size increase further to 27 nm, most of the synthesised NPs were sticking to the magnetic bead used for stirring. They were difficult to separate from the reaction mixture. Because of this, the overall yield of 27 nm IONPs was rather low. To address this problem, we decided to use an overhead stirrer. All the synthesis parameters were similar to the reaction conditions reported in the scheme 3.4 except magnetic stirring. However, instead of getting facets, octahedral IONPs of edge length 19.1 ± 2.3 nm IONPs ($\pm$ denoted standard deviation, $n = 104$) were obtained (**Fig. 3.11**). There are

some reports on the effect of magnetic field during NPs synthesis on their morphology. The change in shape morphology from facets to octahedral could be due to the absence of magnetic field during the synthesis.

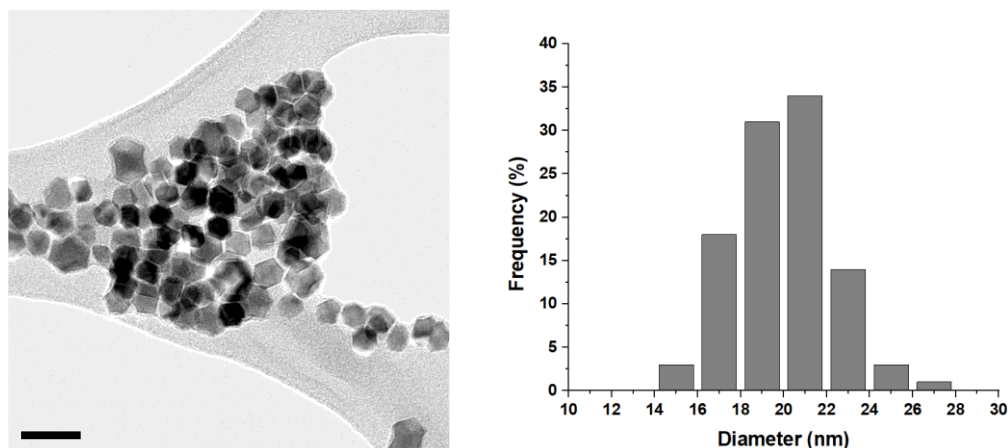
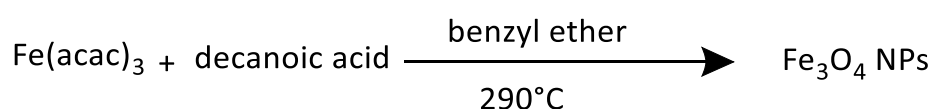


Fig. 3.11. Synthesis of 19 nm octahedral IONPs: TEM image and size distribution histogram analysed by TEM of 19.1 ± 2.3 nm IONPs ($\pm$ denoted standard deviation, $n = 104$).

Optimizing octahedral synthesis procedure to obtain a range of different size IONPs was promising and novel. We could have studied the effect of octahedral morphology on the magnetic heating behaviour of IONPs. However, the main objective of the project was to check the potential use of IONPs for protein delivery applications. Hence, we decided only to study the size effect of IONPs on the triggered protein release. However, studying the effect of other morphologies of IONPs (nanorods, octahedral etc.) on magnetic hyperthermia could be a promising future project.

Due to aforementioned problems with 27 nm NPs synthesis, it was decided to use a different approach to obtain bigger NPs. Iron oxide nanocubes of edge length 33 nm (**Fig. 3.12**) were obtained by refluxing a mixture of $\text{Fe}(\text{acac})_3$ and decanoic acid in benzyl ether³ (**Scheme 3.6**).



Scheme 3.5. Synthesis of 33 nm cubical IONPs by thermal decomposition of $\text{Fe}(\text{acac})_3$. Optimized conditions: mole ratio = $\text{Fe}(\text{acac})_3$: decanoic acid = 1:4, 25 ml benzyl ether, heating rate = $10^\circ\text{C}/\text{min}$ under argon.

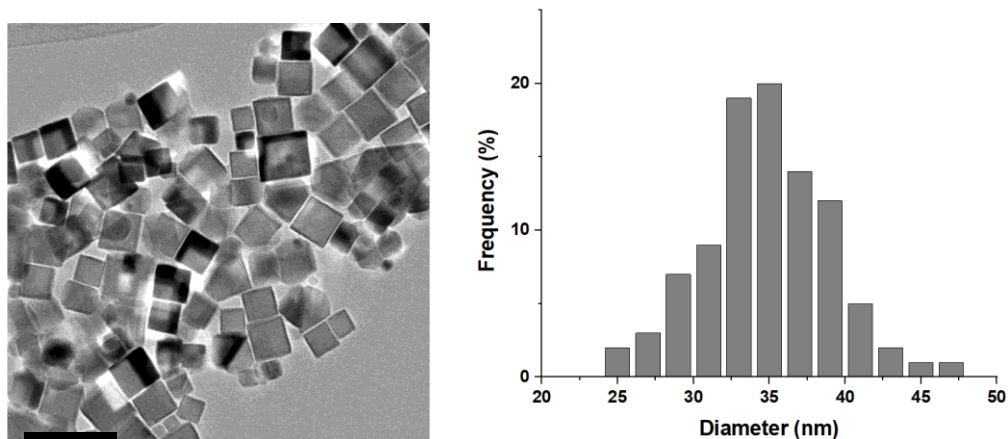
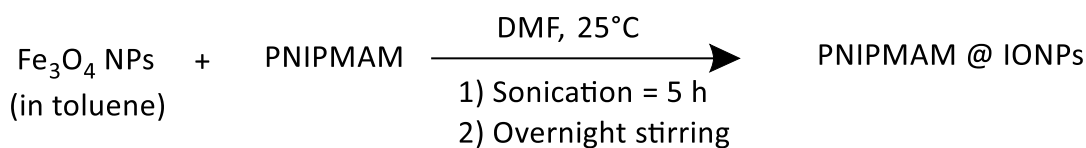


Fig. 3.12. Synthesis of 33 nm cubical IONPs: TEM image and size distribution histogram analysed by TEM of 33.4 ± 4.9 nm IONPs ($\pm$ denoted standard deviation, $n = 98$).

All the NPs synthesized using thermal decomposition of $\text{Fe}(\text{acac})_3$ were soluble in organic solvents and hence stored in toluene. To make them water dispersible, ligand exchange with PNIPMAM was done to remove organic moieties (oleic acid / oleylamine) from NPs surface. The synthesis of these core-shell NPs is reported in the next section.

3.2. IONPs core – PNIPMAM shell NPs

To prepare IONPs core PNIPMAM shell NPs (PNIPMAM@IONPs), the polymer was end functionalized with nitrodopamine (NDA) prior to ligand exchange with NPs.¹⁰⁻¹¹ For ligand exchange, there were two reported methods; film method and ligand exchange method¹⁸. In the film method, NPs suspension (in organic solvent) is mixed with the polymer (in water), and, a film is made by solvent drying which results in transfer of NPs from organic to aqueous phase. In ligand exchange, new ligand (in DMF) and NPs (in toluene) are mixed (sonicated) to move NPs to water.¹⁰⁻¹¹ We tried both methods to obtain water dispersible PNIPMAM @ IONPs. During film method, NPs obtained after solvent drying were agglomerated and difficult to dissolve. Therefore, the ligand exchange method was adapted¹⁰ for the NPs ligand exchange with PNIPMAM (**Scheme 3.6**).



Scheme 3.6. Transfer of IONPs from organic to aqueous phase using ligand exchange method with PNIPMAM. Weight ratio of NPs: PNIPMAM used for ligand exchange of 16 nm = 1:15.

To remove unreacted PNIPMAM, ultracentrifugation at 1600 g-force was done for 1 h at room temperature. After purification, NPs were dispersed and stored in dH₂O at 4 °C. Before moving to study their heating behaviour, a detailed characterization of various PNIPMAM @ IONPs was performed and reported in the next section.

3.2.1. Characterization of PNIPMAM @ IONPs

PNIPMAM-coated IONPs were characterised by determining thermal phase transition and grafting density. These are reported in the next section.

3.2.1.1. Phase transition behaviour of PNIPMAM @ IONPs

After getting stable PNIPMAM @ IONPs suspension, next step was to check the phase transition behaviour of polymer on NPs surface. Nano-differential scanning fluorimetry (NanoDSF) was used as a small-scale turbidimetry method to calculate lower critical solution temperature (LCST) of PNIPMAM @ IONPs. During optimization of measurement conditions, a concentration dependence on the phase transition similar to PNIPMAM was observed. There was an increase in the LCST with the decrease in the Fe concentration of polymer coated NPs (**Fig. 3.13**). However, the concentration dependence on the LCST of PNIPMAM @ IONPs ([Fe]) was much weaker as compared to pure polymer solution ([PNIPMAM]). Apparently, the phase transition in PNIPMAM @ IONPs depends mostly on the interactions between the polymer chains adsorbed on the same particle. This does not depend on concentration, and hence the phase transition of particles depends on the concentration much less than that of the pure polymers.

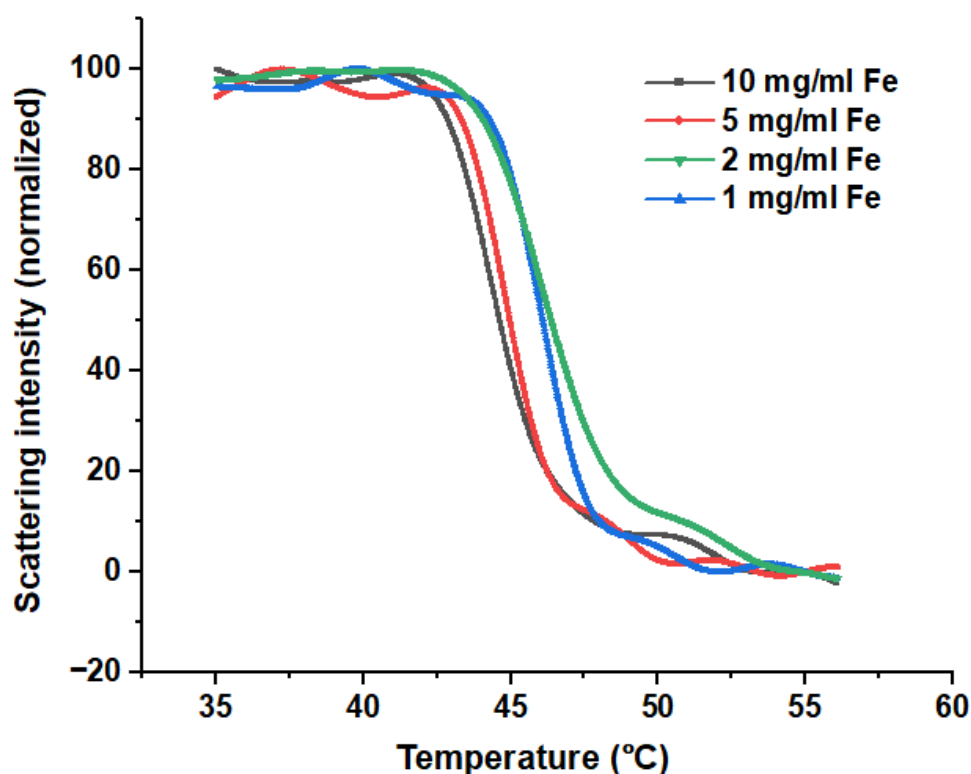


Fig. 3.13. Phase transition studies using NanoDSF: dependence of concentration on LCST for 40 kDa PNIPMAM @ 16 nm IONPs.

Phase transition studies for different chain length PNIPMAM revealed a dependence of LCST on the average molecular weight (M_n) of the polymer. There was a decrease in the LCST with increased M_w of PNIPMAM. However, after coating IONPs with different chain length PNIPMAM, this trend reversed, and, the LCST of PNIPMAM @ IONPs increased with increased chain length of PNIPMAM (**Fig. 3.14**). The phase transition of 7.5 kDa PNIPMAM @ IONPs was broad (36 °C – 50 °C) with no well-defined LSCT. The LCST of 27 kDa PNIPMAM @ 16 nm IONPs was ~ 41.5 °C which increased to ~ 42.8 °C, ~ 44.5 °C and ~ 46 °C for 27 kDa, 40 kDa and 89 kDa PNIPMAM, respectively (**Fig. 3.15**).

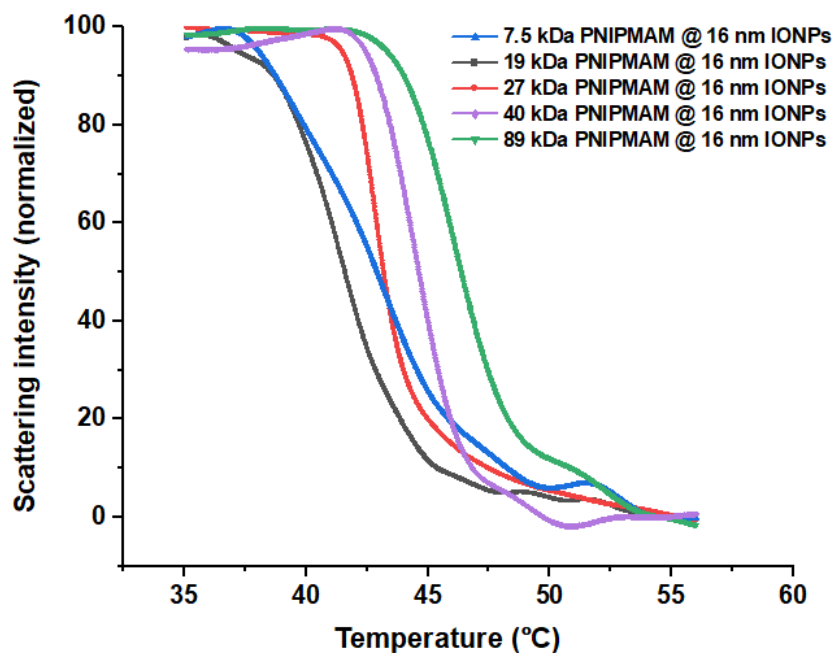


Fig. 3.14. Phase transition studies on PNIPMAM @ IONPs: Phase transition trend after coating different chain length PNIPMAM on 16 nm IONPs. [Fe] = 1 mg/ml.

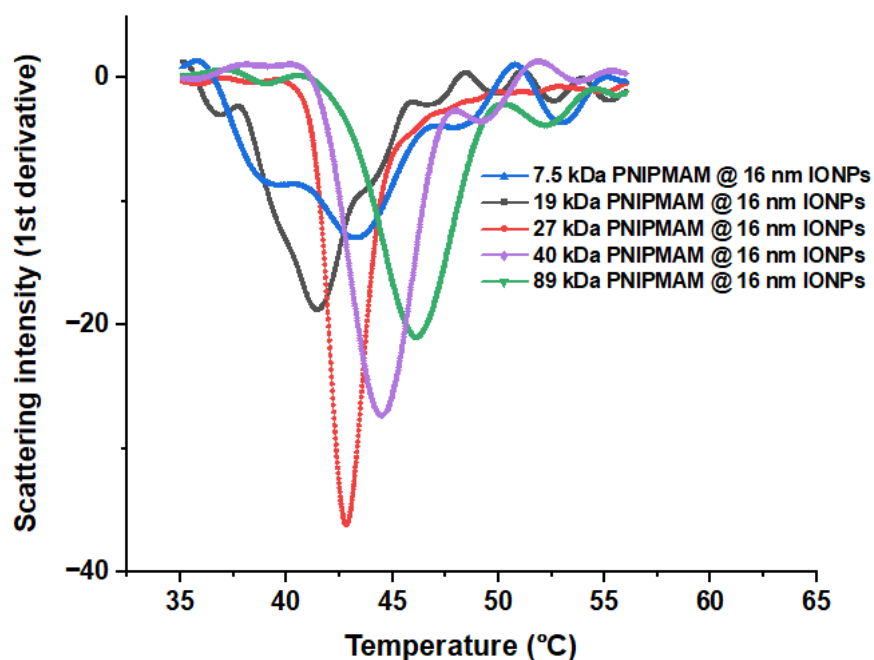


Fig. 3.15. PNIPMAM @ IONPs phase transition study using NanoDSF: Plot of normalized scattering intensity (first derivative) with temperature showing the peak temperature of thermogram. [Fe] = 1 mg/ml.

In the particles coated with shorter polymers, all repeat units from different chains attached to the same particle are close to (and strongly interacting with) each other, essentially resembling a very high molecular weight polymer thus decreasing the LCST. However, for the longer polymer coated NPs, polymer chains protrude further into solution and so behave independently. The chains do not interact with each other thus increasing the LCST. Any core shell NPs having phase transition onset below 40°C can't be used for *in-situ* protein delivery applications, due to possible protein leak at normal body temperature (37 °C). Hence, longer polymers (> 27 kDa) were ideal polymer chain lengths for further studies because of their LCST (43 - 46 °C).

After studying the effect of polymer chain length on the phase transition behaviour of PNIPMAM @ IONPs, the next step was to check polymer grafting density using TGA, which is reported in the next section.

3.2.1.2. TGA of PNIPMAM @ IONPs

To investigate the extent of surface coverage of NPs by polymer, TGA was used as a method of thermally decomposing PNIPMAM on the PNIPMAM @ IONPs surface.¹⁰⁻¹¹ The mass loss from polymer-coated particles following PNIPMAM decomposition was used to deduce the amount of surface bound polymer. To check the effect of NPs size and shape on the grafting density of polymer, 40 kDa PNIPMAM was used with different IONPs. TGA results for different size and shaped 40 kDa PNIPMAM-coated IONPs are shown in **Fig. 3.16**. Weight loss between 300 °C – 450 °C represented the amount of PNIPMAM present on NPs surface. Smaller IONPs would have higher surface area as compared to bigger NPs results in more polymer on their surface. We also observed a gradual increase in the polymer weight loss with decrease in NPs size from 33 nm to 7 nm.

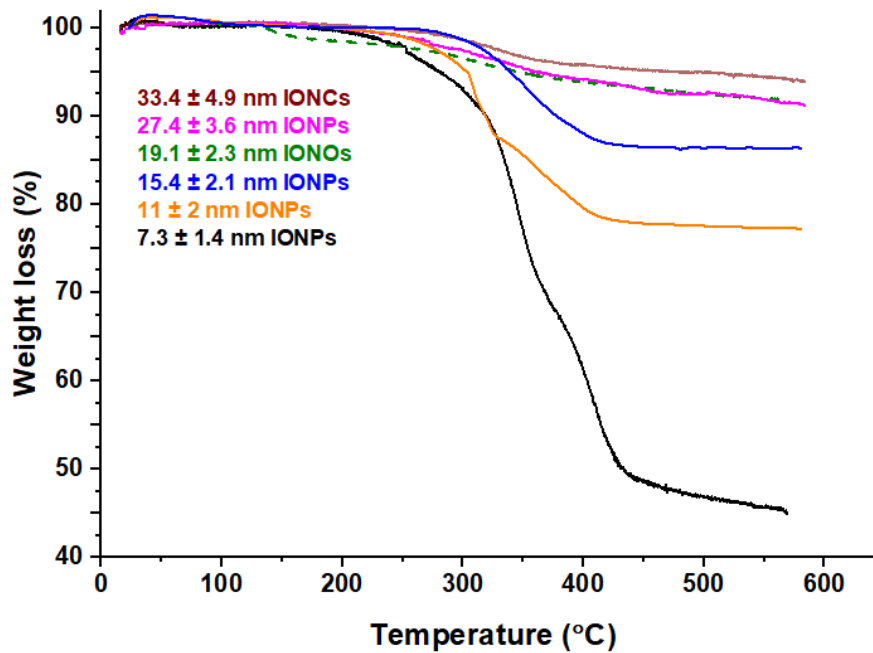


Fig. 3.16. TGA analysis of different size and shaped PNIPMAM @ IONPs. (PNIPMAM = 40 kDa). 20 mg dry PNIPMAM-coated IONPs samples heated under air at a ramp rate of 10 °C min⁻¹ between 0-600 °C.

To calculate the grafting density of various PNIPMAM @ IONPs, this weight loss between 300°C - 450°C has been used. Grafting density calculations for 40 kDa PNIPMAM @ 16 nm IONPs are reported below. Grafting density of polymer on the NP surface can be determined by using equation 3.1.

$$\text{Grafting density} = \frac{\text{Number of PNIPMAM chains on NP}}{\text{Surface area of a NP}} \quad (3.1)$$

The grafting density of PNIPMAM @ IONPs was determined by first calculating NP volume using equation 3.2.

$$\text{Volume of a sphere (V)} = \frac{4}{3}\pi r^3 \quad (3.2)$$

Here, r = radius of a sphere. TEM derived NP radius (8 nm) was used to calculate volume of a 16 nm IONP (diameter, TEM) which was $2.14 \times 10^{-18} \text{ cm}^3$. From volume, the mass of a single NP (M) was determined ($1.11 \times 10^{-17} \text{ g}$) using equation 3.3.

$$\text{Density} = \frac{M}{V} \quad (3.3)$$

Density of bulk iron oxide = 5.18 g/cm³. The mass of the NP with a ligand shell (M') was derived using equation 3.4.

$$M' = \frac{\text{Mass of a spherical NP}}{\text{Inorganic fraction of PNIPMAM @ IONP}} \quad (3.4)$$

Inorganic fraction of 40 kDa PNIPMAM @ 16 nm IONP determined using TGA (between 300 – 450 °C) was 0.86 (86 %). Hence, the mass of NP with a ligand shell was calculated as 1.29 x 10⁻¹⁷ g. The mass of the organic shell in a single nanoparticle (m) was then calculated using equation 3.5.

$$m = M' * \text{organic fraction of PNIPMAM @ IONP} \quad (3.5)$$

Organic fraction of PNIPMAM @ IONP determined by TGA (0.14) was used to calculate PNIPMAM mass (1.8 x 10⁻¹⁸ g). To determine the number of PNIPMAM chains on the NP surface, this mass (m) was divided by the mass of a single PNIPMAM chain which was calculated using equation 3.6.

$$\text{Mass of a single PNIPMAM chain} = \frac{M_n}{N_A} \quad (3.6)$$

Here, M_n = average molecular weight of PNIPMAM = 40 kDa, and N_A = Avogadro's number. Finally, surface area of a spherical NP was determined to be 803.8 nm² by using equation 3.7.

$$\text{Surface area of a sphere} = 4\pi r^2 \quad (3.7)$$

Here, r = radius of sphere (8 nm). Hence, the grafting density of 40 kDa PNIPMAM @ 16 nm IONPs calculated using equation 3.1 was 0.03 chain/nm². The grafting densities calculated for different PNIPMAM @ IONPs were low as compared to our previous report with 10 kDa PNIPAM @ 5 nm IONPs (0.3 chain/nm²).¹¹ The reason for low grafting density with PNIPMAM

could be the steric hindrance due to the α -methyl groups in the polymer repeat units. However, the grafting density values were varied slightly for different chain length PNIPMAM on similar size IONPs (16 nm) and the same chain length polymer (40 kDa) on different size IONPs.

Table 3.2. TGA-calculated grafting density of different 40 kDa PNIPMAM @ IONPs.

Core diameter of 40 kDa PNIPMAM @ IONPs (TEM)	Grafting density (nm ⁻²)
7.3 ± 1.4 nm	0.049
11 ± 2 nm	0.031
15.4 ± 2.1 nm	0.029
19.1 ± 2.3 nm [#]	0.016
27.4 ± 3.6 nm	0.025
33.4 ± 4.9 nm [*]	0.017

Note. # = octahedral and * = cubes.

To validate the TGA findings, another approach was used to assess the amount of polymer on the NPs surface by dissolving 40 kDa PNIPMAM @ 16 nm IONPs (1 ml of 10 mg/ml Fe) in acid (6 M HCl) and separating the Fe²⁺ ions from the solution by dialysis (M_w cutoff 14 kDa). Dialyzed solution was dried by rotary evaporation and the dry mass was weighed (~ 4 mg). This gives 40% organic fraction which contrasts with the TGA results (14%). Presumably the residue was not dry. To substantiate these findings, ¹H NMR of the dry product with an internal standard 2, 4-dinitrochlorobenzene (DNB, 10 mg) was recorded (**Fig. 3.17**).

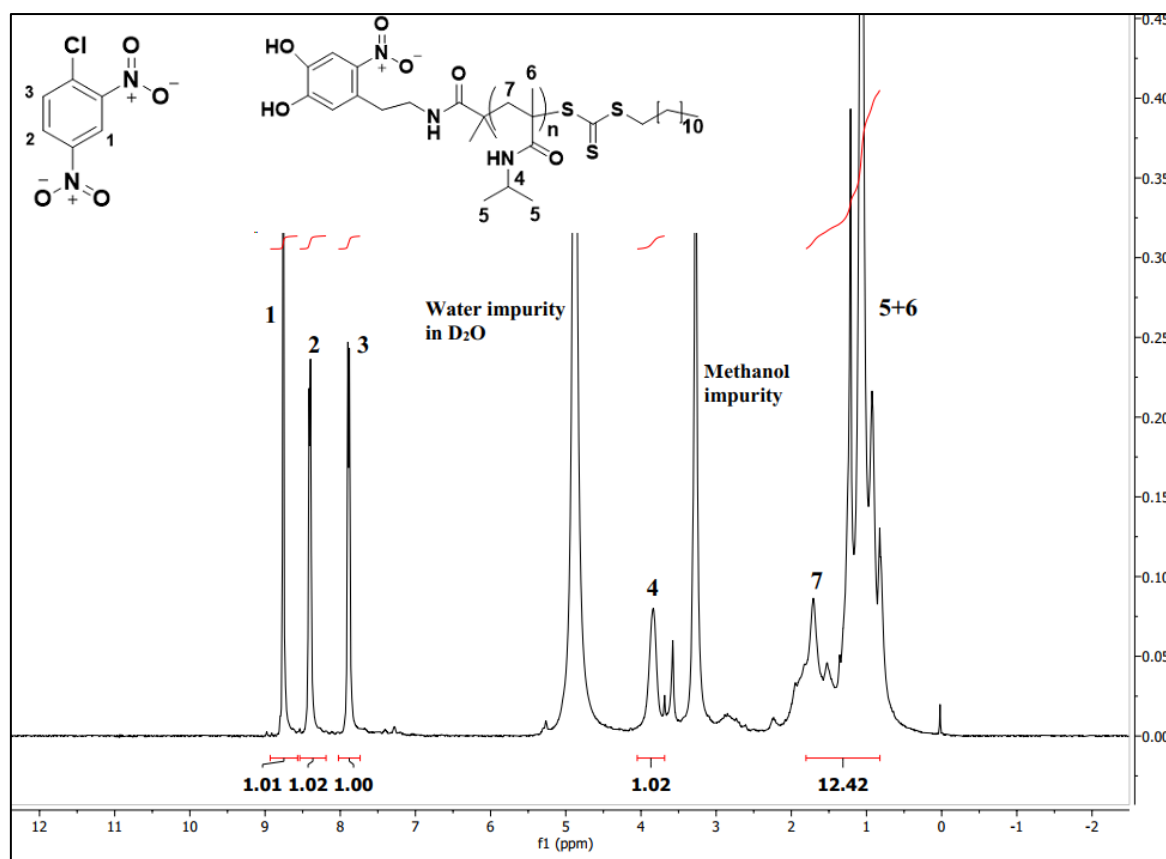


Fig. 3.17. ^1H NMR of the dry mass (~ 4 mg) with 10 mg DNB (0.049 mmol) as internal standard. Since, the integral ratio between aromatic protons of DNB (8 – 9 ppm) and CH protons of PNIPMAM (3.9 ppm) is 1:1, dry mass would have 0.049 mmol of PNIPMAM. It was converted to mass (1.9 mg) by multiplying with PNIPMAM molecular weight (40 kDa).

NMR peaks of PNIPMAM was used to quantify PNIPMAM by comparing the integral with the aromatic peaks of DNB. Polymer content calculated with this approach (19 %) was in accordance with the TGA findings (14 %). Elemental analysis (CHN) on 40 kDa PNIPMAM @ 16 nm IONPs showed 11 % of PNIPMAM on IONPs (**Table 3.3**). In conclusion, polymer content in 40 kDa PNIPMAM @ 16 nm estimated using TGA was in according with the NMR and CHN analysis.

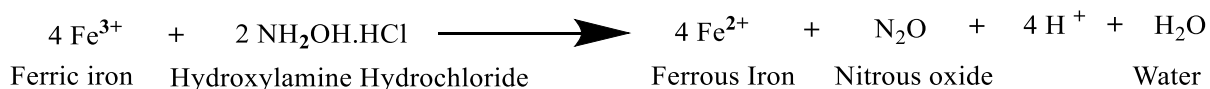
Table 3.3. Elemental analysis of 40 kDa PNIPMAM @ 16 nm IONPs

PNIPMAM@IONPs (40 kDa @ 16 nm)	% C	% H	% N	% remainder
Sample 1	9.2	1.4	0.6	88.8
Sample 2	9.0	1.4	0.6	89.0

For SAR estimation of IONPs, accurate total Fe content of NPs is required (Section 3.2.2.3). TGA could give us a crude idea about the total inorganic content of the NPs (mainly iron oxide) which could be used to estimated total Fe content of the NPs. However, TGA is a destructive technique which requires high sample amounts, and hence was difficult to use frequently. Therefore, we determined the total Fe content in the NPs spectrophotometrically, using the UV-Vis spectrophotometer.⁴⁷ The method is discussed in the next section.

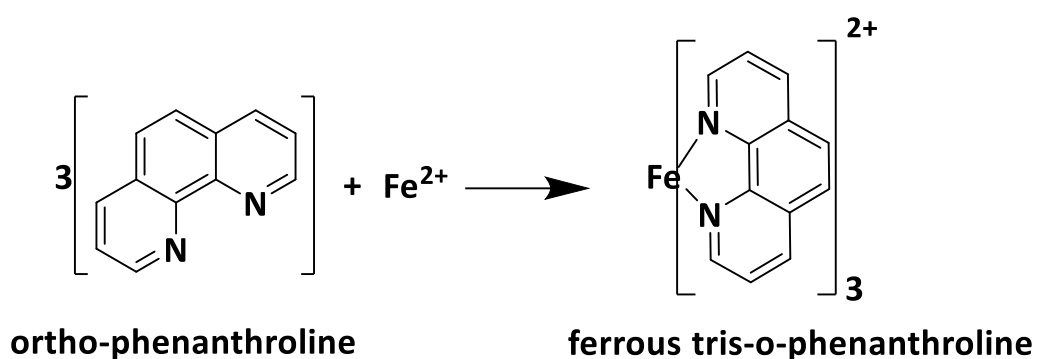
3.2.1.3. Total iron content of IONPs using UV-Vis

The process was started with dissolving the known weight (1 mg, dry) of PNIPMAM @ IONPs in the minimum volume of conc. HCl, resulting in the formation of a solution containing a mixture of Fe²⁺ and Fe³⁺ ions. The resulted solution was then diluted using dH₂O (25 ml) in a 50 ml volumetric flask and all the iron was then reduced to Fe²⁺ by adding excess of hydroxylamine hydrochloride (4 ml, 10 wt% in dH₂O).



Scheme 3.7. Reduction of ferric iron to ferrous iron using hydroxylamine hydrochloride as a reducing agent.

To this Fe²⁺ solution, o-phenanthroline (4 ml, 0.3 wt% in ethanol) was added resulting in the formation of an orange red complex (pH = 6-6.5) and the Fe content was determined using the UV-Vis spectrophotometer.



Scheme 3.8. Reaction of o-phenanthroline with ferrous ion resulted in the formation of ferrous tris-o-phenanthroline (orange red complex).

Ammonium iron(II) sulfate or Mohr salt was used as a stable Fe^{2+} source and a calibration curve (**Fig. 3.18**) was obtained to determine iron concentration of IONPs. For 1 mg of 40 kDa PNIPMAM @ 16 nm IONPs, the total iron content determined by UV method was 0.86 mg (86 %), which was in accordance with the predictions from NPs mass (89 %). Hence UV method was used to determine total Fe content of NPs for the SAR calculations.

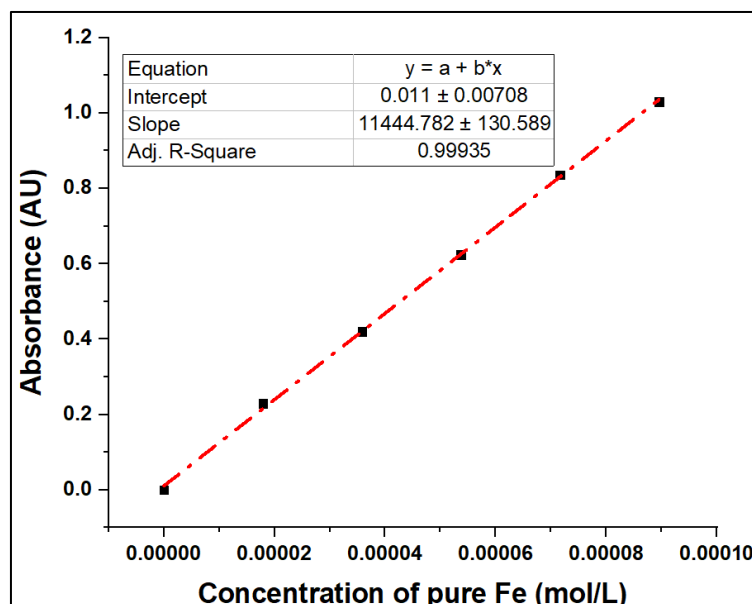


Fig. 3.18. Standard curve to determine total iron content of IONPs ferrofluid.

In the next section, size and surface charge determination of PNIPMAM @ IONPs is discussed in detail.

3.2.1.4. Size and zeta potential measurements of PNIPMAM @ IONPs

Dynamic light scattering (DLS) is a method to estimate the size of the NPs in the aqueous medium. DLS gives us an idea about how a NP would diffuse within a fluid (hydrodynamic diameter) not its actual size. For PNIPMAM @ IONPs, DLS could give us an idea about the overall diameter of the core-shell particle. Hydrodynamic diameter for the 40 kDa PNIPMAM @ 7 nm IONPs was 38 nm, which further increased to 180 nm for the 40 kDa PNIPMAM @ 33 nm IONPs (**Table 3.4**). These values were significantly higher than the estimated diameter of the PNIPMAM @ IONPs (**appendix C**). The polymer chains on the NP surface (in solution) during DLS measurement would be expanded and interacting with other NPs, which would make their diffusion slow. This could result in high hydrodynamic diameter of the polymer-coated NPs than their actual size.

Table 3.4. PNIPMAM @ IONPs: core diameter (TEM), grafting density (TGA), hydrodynamic diameter (DLS) and zeta potential of different 40 kDa PNIPMAM @ IONPs.

Core diameter of 40 kDa PNIPMAM @ IONPs (TEM)	Grafting density (nm ⁻²)	Hydrodynamic diameter (nm, DLS)	Estimated diameter of PNIPMAM @ IONPs (nm) [‡]	Zeta potential (mv)
7.3 ± 1.4 nm	0.049	~ 38	~ 12	-18
11 ± 2 nm	0.031	~ 52	~ 16	-2.6
15.4 ± 2.1 nm	0.029	~ 70	~ 21	-1.9
19.1 ± 2.3 nm [#]	0.016	~ 80	~ 24	-1.8
27.4 ± 3.6 nm	0.025	~ 150	~ 32	-1
33.4 ± 4.9 nm [*]	0.017	~ 180	~ 38	-3.2

Note. All the NPs are faceted except: # = octahedron and * = cubes. ‡ = Calculations are given in **appendix C**.

For triggered protein release from PNIPMAM @ IONPs, proposed mode of action was magnetic heating of the iron oxide core using alternate magnetic field. Hence, the magnetic heating characterization of various PNIPMAM @ IONPs is reported in the next section. Citrate (CA) coated counterparts were used as a benchmark to check the effect of bigger non-magnetic PNIPMAM coating on SAR values of IONPs. After ligand exchange, CA @ IONPs formed a stable suspension (in water) for the smaller IONPs (7, 11 and 16 nm) whereas, the suspension wasn't stable for bigger NPs (19, 27 and 35 nm NPs precipitated in ~ 15 min). Polymer coating improved the stability of bigger NPs but they were still precipitating possibly due to agglomeration (in ~ 1 h). For biomedical applications, as the overall size of the NP system should be less than 150 nm for entry and exit from capillary circulation.²⁶ Therefore, aggregation was not desirable for our study.

3.2.2. Magnetic heating studies IONPs

After preparing NPs suspension in water, magnetic heating studies were done on various ligand coated IONPs. In this section, magnetic heating setup is briefly described followed by the measurements of the SAR values. Finally, magnetic heating characterization of polyol and citrate coated IONPs, followed by various PNIPMAM @ IONPs has been reported.

3.2.2.1. Magnetic heating setup

The heating performance of the water dispersible Fe₃O₄ NPs was measured under an applied alternating current magnetic field (AMF) at a constant voltage (30.0 V) and current limit (1.95 A). All equipment to carry out magnetic heating studies (**appendix D**) of the IONPs was generously provided by Dr. Iain Will, (Department of Electronics, University of York).

Prior to magnetic heating experiments, calibration was conducted by checking background heating of dH₂O in AMF. 0.45 ml dH₂O was loaded to a sample tube of dimensions 8 mm × 40 mm (diameter × height, Sigma) and the sample was put inside the heating chamber. Sample

tube was held in a fixed position by a plastic holder and a lid was adapted to allow for insertion of thin plastic tubing for gallium arsenide thermocouple probe to monitor the temperature changes inside the chamber (**appendix D**). Magnetic heating data for various citrate coated IONPs were recorded using above described procedure.

To quantify and compare magnetic heating strength of different IONPs, heating data were analysed to calculate specific absorption rate (SAR) values. SAR can be determined as a readout of the total energy input in the solution and thereby acts as a value for optimisation of magnetic IONPs for magnetic hyperthermia applications. IONPs with a high SAR value would allow a lower field/frequency AMF to be used to achieve the same amount of heat generation as a sample with a lower SAR value and higher field/frequency. It allows heating within tolerable amplitudes and frequencies of the AMF, thereby reducing the adverse side effects of heating in healthy cells by eddy current.

3.2.2.2. Magnetic heating of polyol and citrate coated IONPs

The AMF strength (28.7 mT) and frequency (102.4 kHz) used in our measurements were very mild (AMF strength x frequency = 2884.2 Ts^{-1}) as compared to the conditions reported in the literature.¹⁻⁴ During the optimization of the heating measurement conditions, we observed a concentration dependence on the magnetic heating for both polyol and citrate coated IONPs. **Fig. 3.19** showed the concentration dependence of citrate coated $15.4 \pm 2.1 \text{ nm}$ IONPs where, there was a gradual increase in heating from $0.8 \text{ }^{\circ}\text{C}/\text{min}$ for $5 \text{ mg}/\text{ml}$ to $1.7 \text{ }^{\circ}\text{C}/\text{min}$ for $10 \text{ mg}/\text{ml}$ of Fe. Heating rate decreased with further increase in concentration to $15 \text{ mg}/\text{ml}$. After 200 s, we observed some background heating of the water sample (control), which could be due to the heat transfer from the coil to the sample. Hence, to calculate actual SAR values IONPs, heating data for first 150 s was used for the further analysis.

The SAR value of IONPs increased from $20 \text{ W}/\text{g}$ to $24 \text{ W}/\text{g}$ with increased Fe concentration from $1 \text{ mg}/\text{ml}$ to $2 \text{ mg}/\text{ml}$. With further increase in Fe concentration, SAR value decreased to $13 \text{ W}/\text{g}$ and $7 \text{ W}/\text{g}$ for $10 \text{ mg}/\text{ml}$ and $15 \text{ mg}/\text{ml}$, respectively (**Fig. 3.20**). These results were in accordance with the literature report by Iqbal *et. al.* where, they reported an increase in the

SAR value with increase in Fe concentration of 17 nm silica @ IONPs (AMF frequency = 260 kHz).²¹

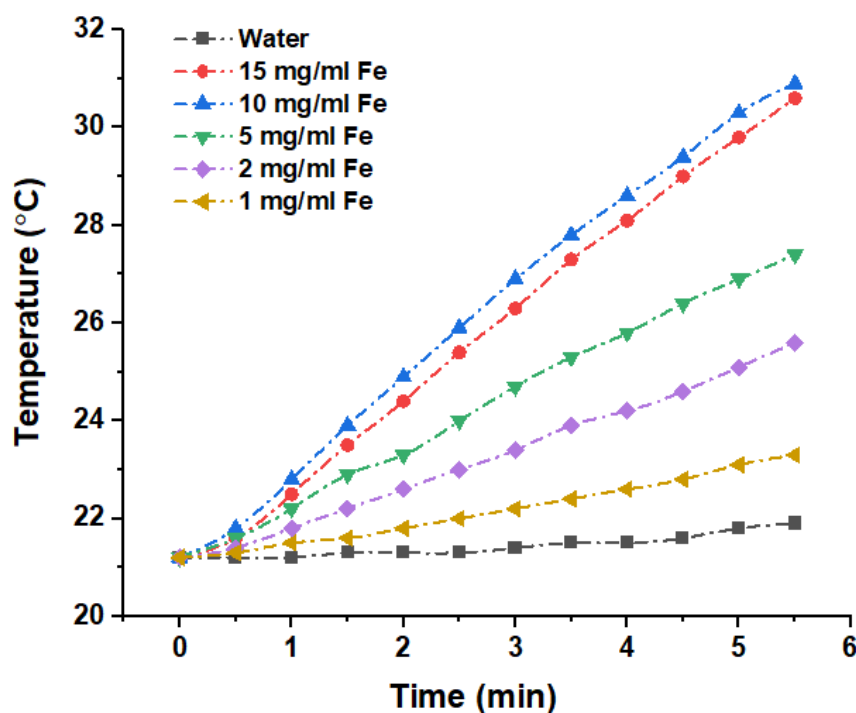


Fig. 3.19. Concentration dependence on the magnetic heating of IONPs: Different concentrations of 16 nm citric acid @ IONPs, AMF strength of 28.7 mT and frequency of 102.4 kHz. AMF strength x frequency = 2884.2 Ts⁻¹.

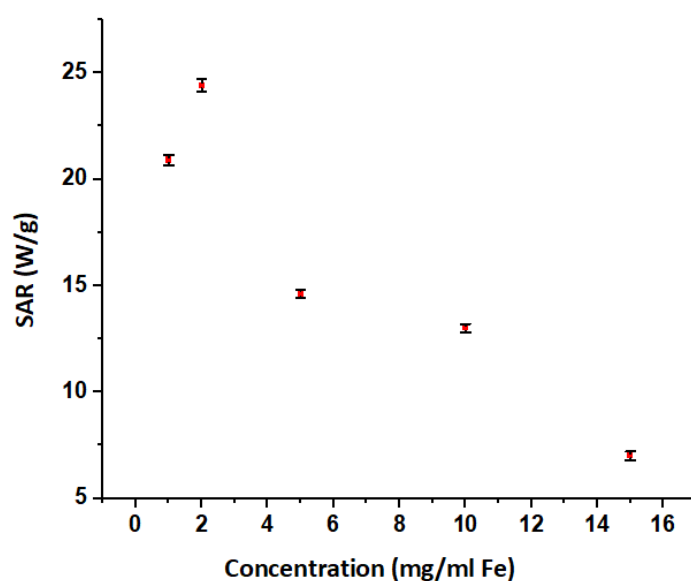


Fig. 3.20. Concentration dependence on the SAR values of 16 nm citric acid @ IONPs (AMF strength = 28.7 mT and frequency = 102.4 kHz). (n = 3, error bars denote standard error).

Because of high heating rate of the 10 mg/ml IONPs (16 nm), similar concentration condition was used for measuring the SAR of various NPs. **Fig. 3.21** shows magnetic heating curves of various polyol and citric acid coated IONPs (10 mg/ml). The SAR values obtained for polyol and citrate coated IONPs are reported in the table 3.5.

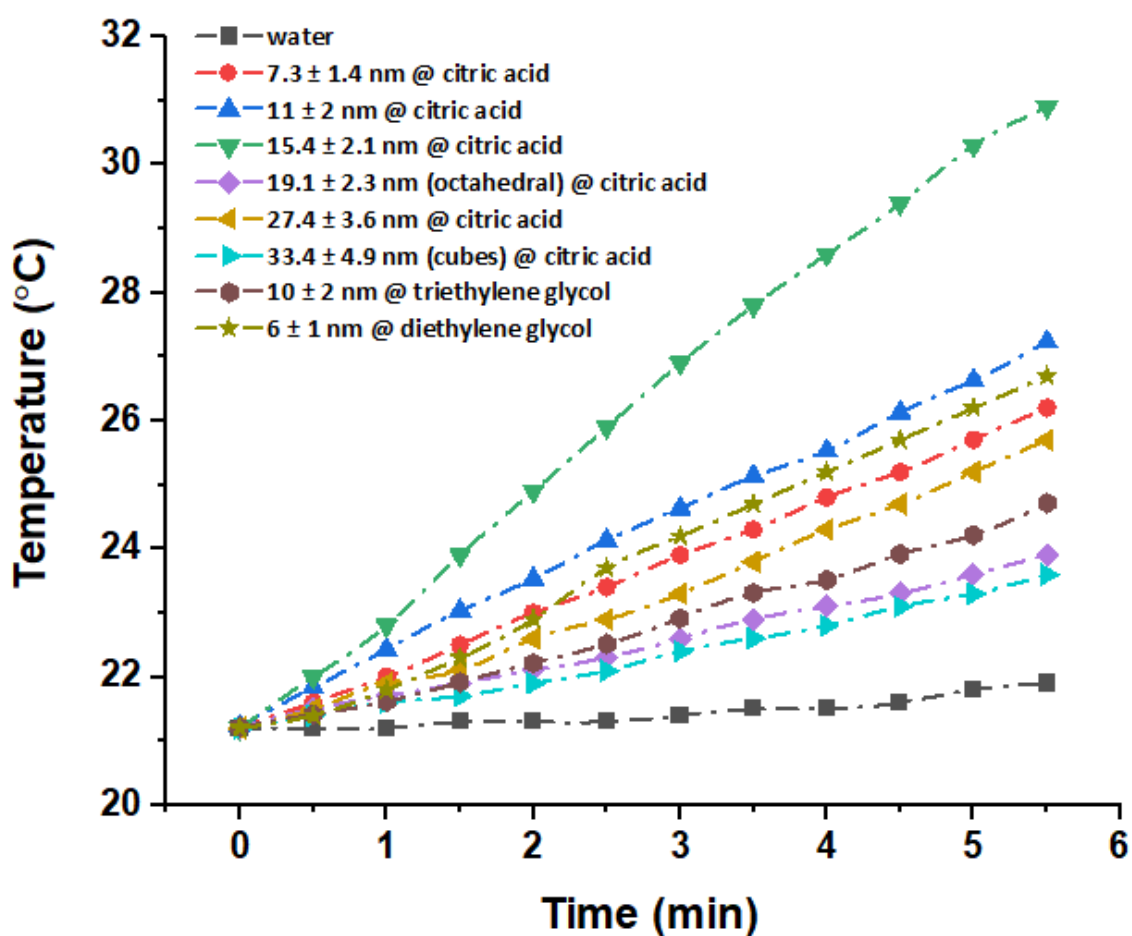


Fig. 3.21. Heating curve analysis of different size and shaped water dispersible IONPs. Effect of different ligands on NPs surface on their magnetic heating behaviour is also reported here (polyols and citric acid). Measurement conditions: 0.45 ml of 10 mg/ml ferrofluid, AMF strength of 28.7 mT and frequency of 102.4 kHz).

With polyol coated IONPs, the SAR value of 6 nm IONPs @ DEG was 7.0 W/g (1.0°C/min). With further increase in size to 10 nm using longer chain TREG, SAR value dropped to 3.6 W/g (0.6°C/min). IONPs synthesized using higher chain length TREG and TEG were precipitating over time (~ 30 min). Hence, this decrease in SAR could be due to agglomeration of TREG coated NPs. However, citric acid coated IONPs synthesized using thermal decomposition of Fe(acac)₃ showed an increase in SAR from 8.08 W/g (1.1 °C/min) for 11 nm NPs to 13.11 W/g (1.8 °C/min) for 16 nm IONPs. With further increase in size, SAR decreased to 4.7 W/g (0.8 °C/min) for 27 nm (4.7 W/g) and 3.06 W/g (0.4 °C/min) for 33 nm nanocubes. 19 nm octahedral NPs showed poor heating performance with the SAR value of 2.5 W/g (0.5 °C/min).

Table. 3.5. SAR values of various size ligand @ IONPs of various size and shaped IONPs

Core diameter IONPs (TEM)	Surface ligand	SAR (W/g)
6 ± 1 nm	diethylene glycol	7.0
7.3 ± 1.4 nm	citric acid	6.14
10 ± 2 nm	triethylene glycol	3.6
11 ± 2 nm	citric acid	8.08
15.4 ± 2.1 nm	citric acid	13.11
19.1 ± 2.3 nm [#]	citric acid	2.5
27.4 ± 3.6 nm	citric acid	4.7
33.4 ± 4.9 nm [*]	citric acid	3.06

Note. All the NPs are faceted except: # = octahedral and * = cubes. Measurement conditions: 10 mg/ml, AMF strength of 28.7 mT and frequency of 102.4 kHz).

In a similar study, Mohapatra *et. al.* observed that SAR increases with the increase in particle size and attains a maximum at a particle size of 28 nm, then the value decreases with a further increase in the particle size to 40 nm.¹ We observed similar size behaviour on magnetic heating of citric acid @ IONPs where, the SAR values increased with increase in NPs diameter to 16

nm. With further increase in size, there was a decrease in the SAR, which could be due to the formation of aggregates during ligand exchange.

After the ligand exchange with citric acid, NPs bigger than 16 nm were forming stable NPs suspension. However, during their heating measurement, some bigger aggregates were observed to attract towards the magnetic core. Therefore, these aggregates wouldn't be involved in their magnetic heating, which could explain their lower heating performance than 16 nm IONPs. This problem could be addressed by using a bulkier ligand such as PNIPMAM which would form a physical barrier on NPs surface preventing their agglomeration. In the next section, heating efficiency of various PNIPMAM @ IONPs has been described followed by a brief summary of this chapter.

3.2.2.3. Magnetic heating studies of PNIPMAM @ IONPs

As expected, bigger NPs (> 16 nm) after polymer coating were stable, with no aggregates attracted to the magnetic core during heating measurement. Additionally, the heating rate (SAR) of PNIPMAM @ IONPs (up to 16 nm) was lower as compared to their citric acid counterpart. This was expected as the non-magnetic PNIPMAM shell would decrease the heating efficiency of IONPs. Similar size trend to the citric acid coated NPs was observed (**Fig. 3.22**) where, the SAR value increased from 4.2 W/g (7 nm) to 7.5 W/g (16 nm), which then gradually decreased (6.13 W/g for 27 nm NPs) with further increase in size (3.9 W/g for 33 nm cubes). Higher SAR values than for citric acid @ IONPs for the 27 nm and 33 nm PNIPMAM @ IONPs was indicating the possible agglomeration in citric acid coated NPs (**Table. 3.6**).

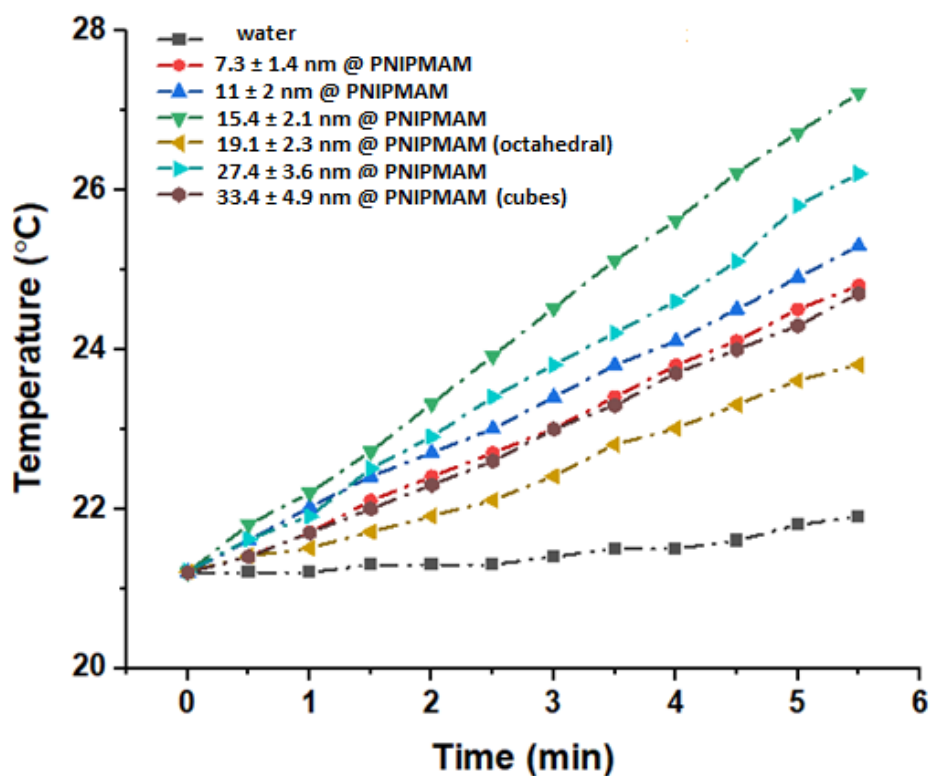


Fig. 3.22. Heating curve analysis of different size and shaped 40 kDa PNIPMAM @ IONPs. Measurement conditions: 0.45 ml of 10 mg/ml ferrofluid, AMF strength of 28.7 mT and frequency of 102.4 kHz).

Table. 3.6. SAR values of various PNIPMAM @ IONPs

Core diameter IONPs (TEM)	SAR (W/g) (citric acid coated)	SAR (W/g) (PNIPMAM coated)
$6 \pm 1 \text{ nm}^+$	7.0	3.1
$7.3 \pm 1.4 \text{ nm}$	6.14	4.2
$11 \pm 2 \text{ nm}$	8.08	5.0
$15.4 \pm 2.1 \text{ nm}$	13.11	7.5
$19.1 \pm 2.3 \text{ nm}^\#$	2.5	2.5
$27.4 \pm 3.6 \text{ nm}$	4.7	6.13
$33.4 \pm 4.9 \text{ nm}^*$	3.06	3.9

Note. + = diethylene glycol coated, # = octahedral and * = cubes. Measurement conditions: 20 mg/ml, AMF strength of 2.2 mT and frequency of 135.7 kHz).

3.3. Conclusions

Following synthesis and characterisation of different IONPs, a method for making optimized PNIPMAM @ IONPs was developed. Stable NP suspension after polymer coating was obtained up to 16 nm IONPs whereas, precipitation was observed in the suspension of bigger NPs (diameter ≥ 19 nm) with in ~ 1 h, possibly due to agglomeration. Magnetic heating characterization of various PNIPMAM @ IONPs revealed the effect of core-size on SAR, where, 16 nm IONPs showed maximum SAR (9.2 W/g). NP aggregation was not desirable for this study and hence PNIPMAM @ IONPs with core diameter ≤ 16 nm (7 nm, 11 nm and 16 nm) were selected for further studies on the magnetically triggered protein release.

3.4. References

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Chapter 4: Gel diffusion studies of PNIPMAM @ IONPs

4.1. Introduction

Hydrogels are viscoelastic materials with porous structure and confined environments.^{1,2} The network structure of hydrogels resembles those of biological media, such as mucus, extracellular matrices and actin networks.⁶ Because of these similarities, NP-hydrogel composites are an attractive model system for understanding the complexities of NP transport through biological networks.^{4,5} A number of recent studies have begun to look at how diffusion is affected by changes in the composition and surface chemistry of the NPs, which directly affects the chemical and physical properties of NPs.⁷⁻¹⁰ Ligands not only impact the NP surface chemistry, but also add an additional level of complexity to their diffusive behaviour by changing the hydrodynamic diameter and introducing ligand-matrix effects.¹¹ For our studies, we were interested in determining whether PNIPMAM @ IONPs would interact with the polysaccharides in the biological media. If the NPs interact with the gel fiber, they would interact similarly with the glycans on the glycoproteins, which would aid in protein encapsulation/release. Hence, it was decided to study the diffusion of NPs through agar - based hydrogels (agar-agar and agarose, **Fig. 4.1**).

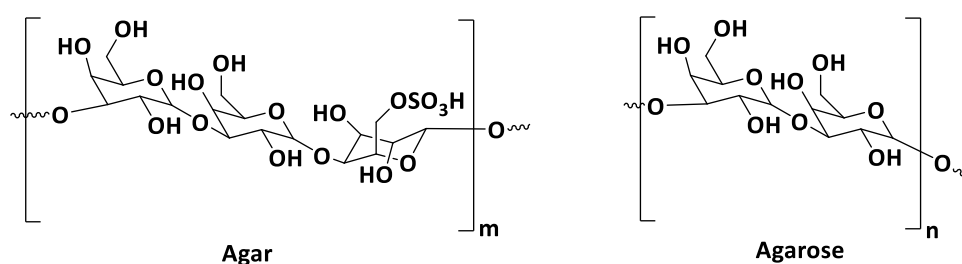


Fig. 4.1. Structural resemblance between agar and agarose

The study of NP diffusion in gels is complicated by the heterogeneous distribution of cross-links within typical hydrogels. Gel mesh size is the physical distance between cross-links and is dependent on the swelling of the material as well as its molecular structure.⁶ Hydrogels typically contain areas of both high and low cross-linking density which makes it difficult to define a single mesh size reflective of the hydrogel as a whole. Moncure *et. al.*⁶ used elastic

blob model¹⁵ for estimating the gel mesh size that particles “see” as they diffuse through the gel. We also estimated the gel mesh size using the elastic blob model^{1,6} where, the mesh size of 0.5 wt% agar hydrogel (30.2 nm, **appendix E**) was well suited for our NP core-size range (7-33 nm).

In the next section, theoretical background for analysing Brownian diffusion, and calculating the diffusion coefficient (D) of NP in a hydrogel is discussed. Subsequently, the experimental findings on the diffusion of IONPs through agar and agarose hydrogels are reported.

4.2. Analyzing Brownian diffusion of IONPs through gel model

4.2.1. Theoretical background

Brownian diffusion arises due to the thermodynamically-driven, passive migration of a species from high to low concentration region, down a concentration gradient ($\delta C/\delta x$). It can theoretically be described by Fick’s second law¹² (**equation 4.1**), which relates partial differentials describing the rate of change of concentration with respect to time ($\delta C/\delta t$) and the rate of change of the concentration gradient with respect to displacement ($\delta^2 C/\delta x^2$).

$$\frac{\delta C(x, t)}{\delta t} = D \frac{\delta^2 C(x, t)}{\delta x^2} \quad (4.1)$$

The diffusion coefficient (D) is a proportionality constant linking the partial differentials and is an intrinsic property of the diffusing species. A higher value of D constitutes rapid diffusion of NPs through a material (not necessarily a gel). In the biomedical context, this implies more efficient migration through the interstitium and more efficacious delivery to cells.

In our experiment, NPs were loaded onto the gel at $t = 0$ and their downward migration x was measured at regular intervals over a 60-hour time period (**Fig. 4.2**). Through application of the appropriate (t) solution of Fick’s Second Law, diffusion coefficients may be experimentally determined via non-linear fitting.

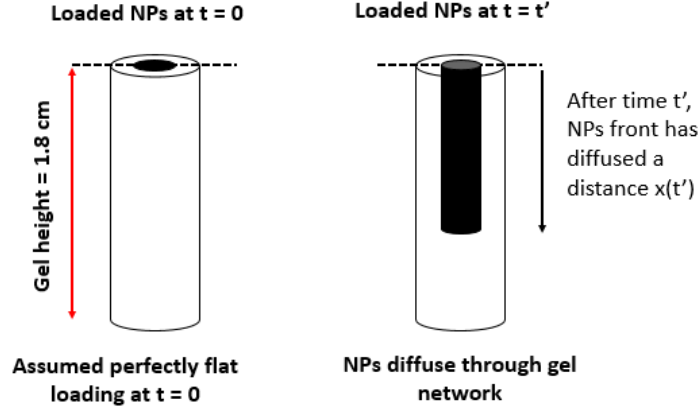


Fig. 4.2. Loading of IONPs onto the gel surface, defined at $x = 0$ and later, their diffusion by a distance (t') from the origin at a time $t = t'$.

Migration of a species through a stationary matrix in the downward x direction is an example of one-dimensional, unidirectional interdiffusion.⁷ Through setting the appropriate boundary conditions, the following solution for $C(x, t)$ was obtained (**equation 4.2**).

$$C(x, t) = \frac{N}{\sqrt{\pi Dt}} \exp\left(\frac{-x^2}{4Dt}\right) \quad (4.2)$$

This can be rearranged, yielding equation 4.3, which has been used to calculate diffusion coefficient of NPs in the gel model.

$$x(t) = \sqrt{4Dt \cdot \ln \frac{N}{C(x, t) \cdot \sqrt{\pi Dt}}} \quad (4.3)$$

The diffusion coefficient thus determined can be compared to the theoretical values calculated using Stokes-Einstein equation for the diffusion of spherical particles (**equation 4.4**).

$$D = \frac{k_B T}{6\pi\eta r} \quad (4.4)$$

Here, k_B is Boltzmann constant, T is the absolute temperature and r is the radius of a particle. Dynamic viscosity of the solvent (η) is the resistance to the movement of one layer of a fluid over another. The hydrogel is almost purely water and so the diffusion through the solvent pools in the absence of NP-gel fibre interactions should be determined by the dynamic viscosity of water (8.9×10^{-4} Pa. s). Radius of a polymer-coated IONP in solution is calculated from its hydrodynamic diameter (**appendix F**) using dynamic light scattering (DLS), and, is used to obtain its theoretical D (D_{Theo}). For 40 kDa PNIPMAM @ 15.4 ± 2.1 nm (hydrodynamic diameter = 38 nm), D_{Theo} calculated using equation 4.4 was 7.0×10^{-12} m²/s.

4.2.2. Studying the migration of aqueous IONPs through hydrogel

For the preparation of hydrogels and setting a gel diffusion experiment, refer to chapter 6 (material and methods). For the diffusion experiment, NPs dissolved in a small volume of solvent (10 mM TRIS, pH 7) were carefully loaded onto the center of the gel surface. Vials were transferred onto the stage (**appendix G**) as soon as the NPs were loaded and the images were recorded at regular intervals for 60 h. Obtained images were analysed using the Gel_Diffusion2.exe software, written by Dr Victor Chechik at The University of York. This software tracked the displacement-time ((t)) distribution of the front of the migrating nanoparticle aliquot by thresholding (above) appropriately (**appendix H**). Lines were drawn on the first recorded image ($t = 0$ seconds) to denote the distance between the bottom of the loaded nanoparticles (on the gel surface) and the bottom of the vial/gel.

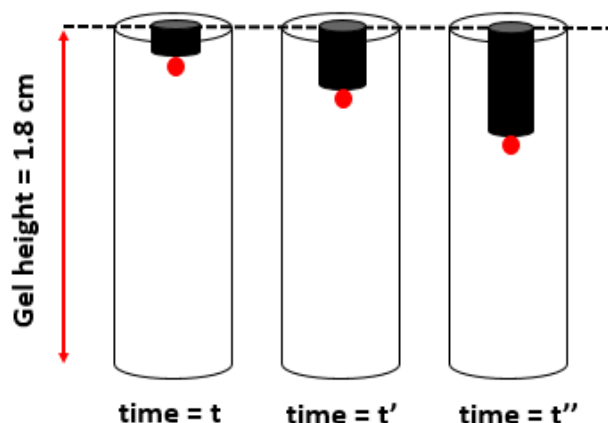


Fig. 4.3. Image processing using Gel_Diffusion2.exe software for the generation of (t) curves.

Images were then cycled through, with the software automatically tracking the ‘relative height’ of the NPs – the ratio of the distance between the nanoparticle front from the gel surface to the total length of the gel (0.018 m) – by thresholding. The dots (**Fig. 4.3**) indicated the measured position of the nanoparticle front at different times in the experiment, and were carefully monitored to ensure that the position assigned by thresholding accurately reflected the observed reality (**appendix H**).

Following this proofing, (t) plots were generated and - where appropriate - fitted to the appropriate non-linear solution (equation 3) of Fick’s Second Law using Origin 2021 software (**Fig. 4.4**). The value of D was obtained from the fitting (**Fig. 4. 4**). For 40 kDa PNIPMAM @ 15.4 ± 2.1 nm (hydrodynamic diameter = 70 nm), experimental D (D_{exp}) obtained after the non-linear curve fitting of the diffusion data was $9.8 \times 10^{-13} \pm 2.3 \times 10^{-13} \text{ m}^2/\text{s}$ which was somewhat lower compared to the D_{Theo} ($7.0 \times 10^{-12} \text{ m}^2/\text{s}$).

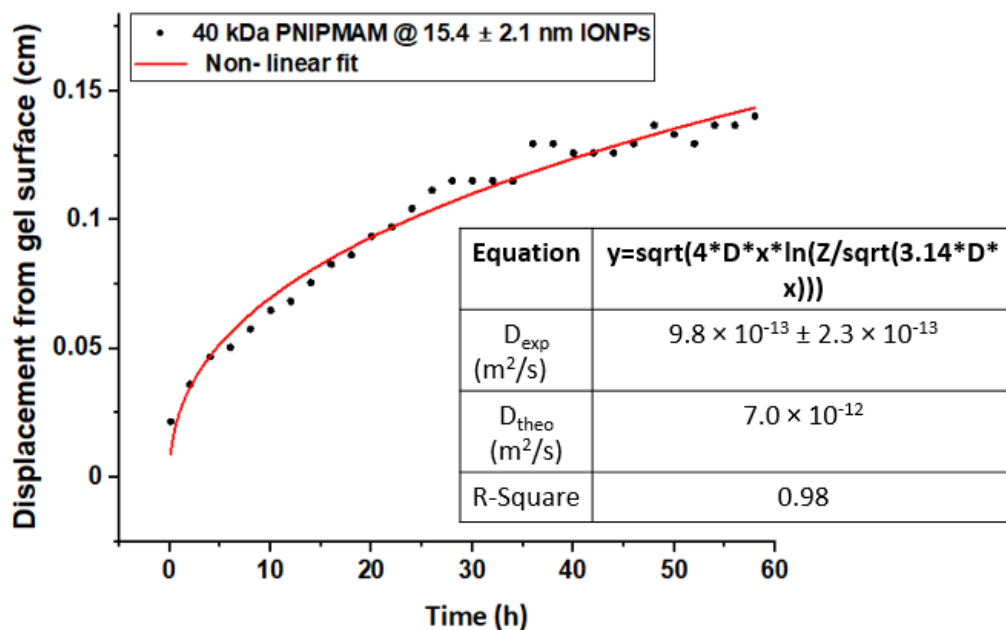


Fig. 4.4. (*t*) plot describing the Brownian diffusion of 5 mg/mL PNIPMAM@IONPs (15.4 ± 2.1 nm) through 0.5 wt% agar gel, fitted to the appropriate non-linear solution of Fick's 2nd Law ($R^2 = 0.98$).

The discrepancy could be due to changes in the dynamic viscosity of the hydrogel, which might vary if the significant amount of agar dissolves in the solvent pools. Moncure *et. al.*⁶ reported similar discrepancies between D_{exp} (PEG @ Au NPs) and Stokes-Einstein D_{theo} . However, the diffusion coefficient decreased with increase in hydrodynamic diameter of Au NPs, in the same trend as predicted by Stokes-Einstein. They further developed more complex models for studying the effect of gel mesh size and hydrodynamic radius on the diffusion of Au NPs. Since the main objective of our study was to monitor the NP-gel fibre interactions as a mimic for the NP-polysaccharide interactions in the biological media, this simple model is sufficient.

If we consider the hydrodynamic diameter (70 nm, DLS) of PNIPMAM @ IONPs and the gel mesh size (~ 30.2 nm), the polymer-coated NP shouldn't diffuse through the hydrogel. Therefore, the hydrodynamic diameter of polymer-coated NP was not accurate. For 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONP, thickness of a packed polymer shell on the NP core was estimated ~ 2.5 nm (**appendix C**). If the polymer shell is packed, the total particle diameter

will be $15.4 + 2 \times 2.5 = 20.4$ nm which is just below the mesh size.

These calculations can be compared with polymer contour length which represents the length of a polymer chain in a maximally elongated conformation. It can be estimated by dividing M_w of the polymer by the M_w of the repeat unit (127 g/mol) and then multiplying by the length of a repeat unit (**equation 4.5**).

$$\text{Polymer contour length} = \frac{M_w \text{ of polymer}}{M_w \text{ of a repeat unit}} \times \text{length of a repeat unit} \quad (4.5)$$

If we assume the length of a repeat unit (monomer length) to be 0.25 nm,¹⁶ the contour length of a single polymer chain would be 78.5 nm (40 kDa PNIPMAM), which is significantly bigger than the estimated thickness of compact PNIPMAM shell on the NP surface (~ 2.5 nm). Perhaps the polymer chain is wrapped around the NP forming a core-shell structure of a diameter of ~ 20.4 nm.

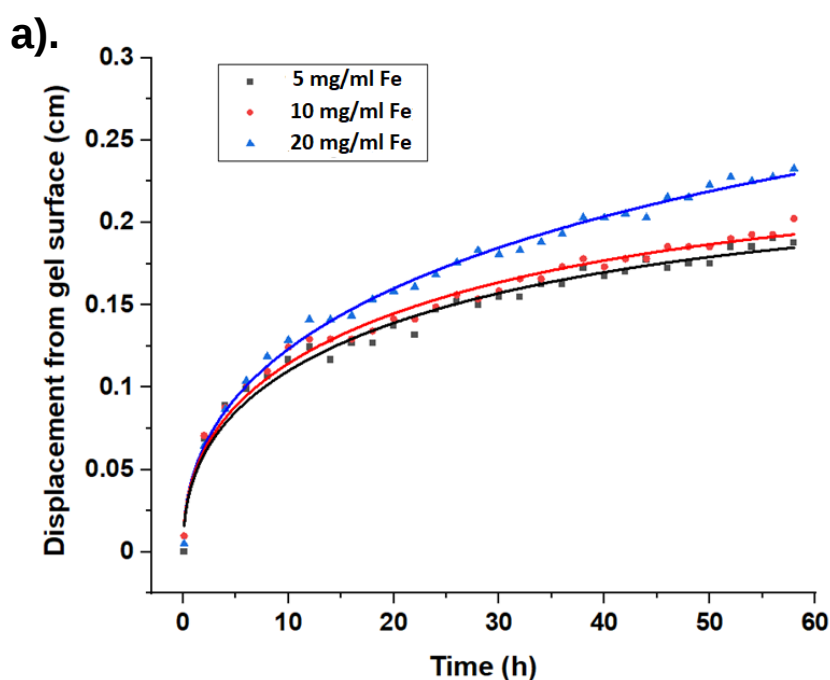
Weak interactions between NPs and gel fibres could contribute to the lower diffusion and hence cannot be ruled out. To substantiate it further, effect of the NP concentration, size and surface ligand on NP diffusion was studied and reported in the following sections.

4.3. Diffusion of IONPs in agar gel

4.3.1. Effect of IONP concentration on diffusion

In Fickian diffusion, particles migrate independently of each other without any positive or negative cooperative effects. Hence, the diffusion coefficient should be independent of the particle concentration. Upon studying the diffusion of three different concentrations of 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs through 0.5 wt% agar gel, slightly different values of maximum displacement from the gel surface were obtained (**Fig. 4.5 (a)**). This could be due to the errors in the NPs diffusion tracking. There is a finite limit to the number of NPs per pixel at which the color can still be detected. Despite having the same overall $C(x)$ profile and D , higher concentration of NPs would reach this limit at a greater percentage through the $C(x)$

distribution. The D_{exp} values obtained after non-linear fitting of the diffusion data for various NPs concentrations (5 mg/ml Fe = $9.8 \times 10^{-13} \pm 2.3 \times 10^{-13} \text{ m}^2/\text{s}$, 10 mg/ml Fe = $9.6 \times 10^{-13} \pm 2.9 \times 10^{-13} \text{ m}^2/\text{s}$, and, 20 mg/ml Fe = $1.04 \times 10^{-14} \pm 2.4 \times 10^{-15} \text{ m}^2/\text{s}$) were similar (within error). Hence, the diffusion of IONPs in agar hydrogel wasn't concentration dependent or was Fickian in nature. 5 mg/ml Fe was selected as the optimal NP concentration for further experiments.



b).

[Fe] of 40 kDa PNIPMAM @ 16 nm IONPs	D_{exp} (m^2 / s)
5 mg/ml	$9.8 \times 10^{-13} \pm 2.3 \times 10^{-13}$
10 mg/ml	$9.6 \times 10^{-13} \pm 2.9 \times 10^{-13}$
20 mg/ml	$1.04 \times 10^{-14} \pm 2.4 \times 10^{-15}$

Fig. 4.5. a) Collated $x(t)$ curves and **b)** a table containing experimentally determined diffusion coefficients for the Brownian diffusion of 20 mg/ml, 10 mg/ml and 5 mg/ml of 40 kDa PNIPMAM @ $15.4 \pm 2.1 \text{ nm}$ IONPs through 0.5 % agar gel (pH = 7). Theoretical $D = 7.0 \times 10^{-12} \text{ m}^2/\text{s}$ ($R^2 > 0.98$).

In order to determine, whether, IONPs diffusion follows the same trend between the diffusion coefficient and hydrodynamic diameter predicted by Stokes-Einstein equation, size dependence on the Brownian diffusion was studied and reported in the next section.

4.3.2. Size dependence of Brownian diffusion of IONPs through agar gel

To further confirm the Fickian nature of diffusion and the absence of NP-gel fibre interactions, the diffusion of different size PNIPMAM @ IONPs through 0.5 % agar gel was studied (Fig. 4.6). Diffusion coefficients were subsequently determined by the non-linear fitting using Equation 3 to the obtained (t) data. High correlation ($R^2 > 0.94$) to the fitting was observed (Table 4.1) in all cases (except 33.4 ± 4.9 nm IONPs). 40 kDa PNIPMAM @ 7.4 ± 1.4 nm IONPs (hydrodynamic diameter = 38 nm) exhibited the maximum displacement from the gel surface with the D_{exp} of $2.0 \times 10^{-12} \pm 5.3 \times 10^{-13}$ m²/s. With further increase in the NPs diameter, NPs diffused slower through the hydrogel. For 33.4 ± 4.9 nm IONPs, following initial displacement of NPs through the gel, NPs diffusion apparently stopped after ~ 12 h. Decreased D with increased particle size is consistent with Fickian diffusion and the absence of strong interactions between the particles and the gel fibres.

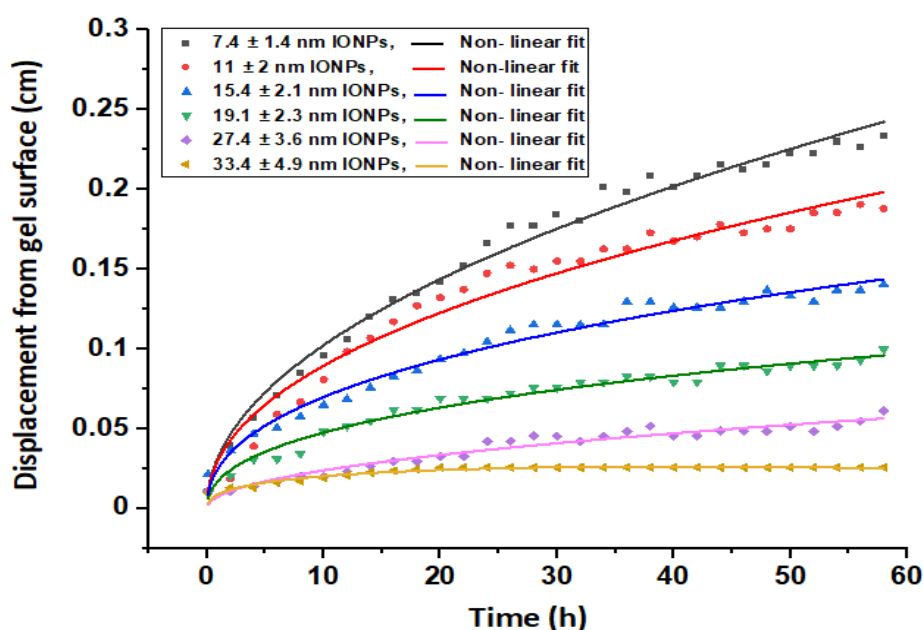


Fig. 4.6. Collated (t) curves with non-linear curve fitting ($R^2 > 0.94$) for the Brownian diffusion of various PNIPMAM @ IONPs through 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml.

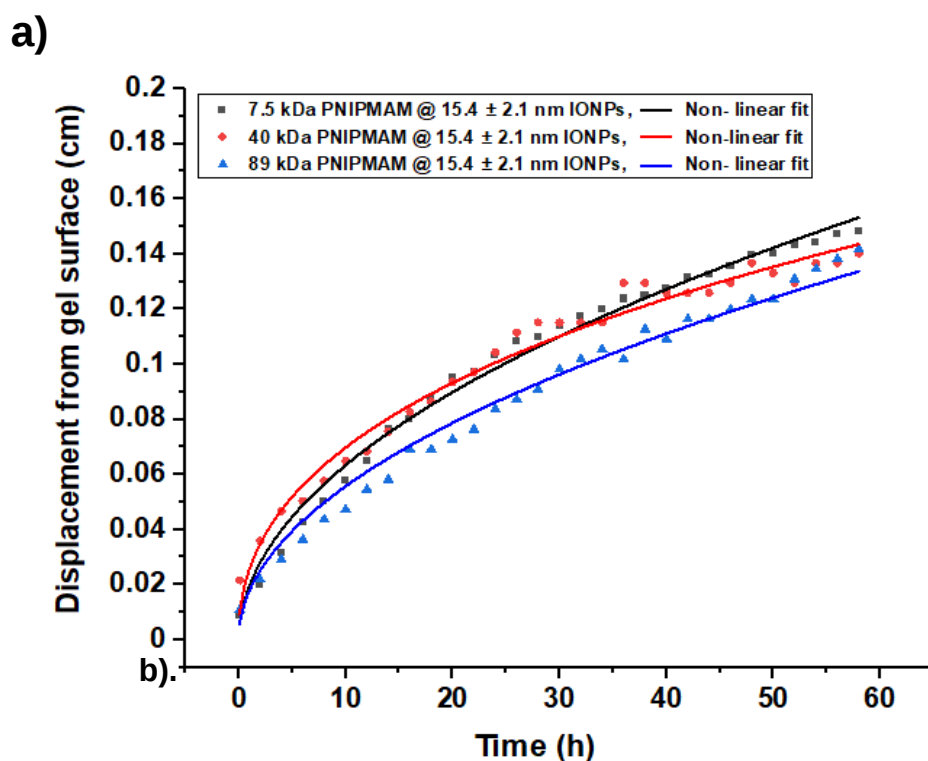
Table 4.1. Experimentally determined diffusion coefficients of 40 kDa PNIPMAM @ 7, 11, 16, 19 and 27 nm IONPs determined by non-linear curve fitting of the $x(t)$ curves for the Brownian diffusion. 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml.

40 kDa PNIPMAM @ 16 nm IONPs (nm, TEM)	Hydrodynamic diameter (nm, DLS)	D_{exp} (m^2 / s)	D_{theo} (m^2 / s)
7.3 ± 1.4	38	$2.0 \times 10^{-12} \pm 5.3 \times 10^{-13}$	1.3×10^{-11}
11 ± 2	52	$1.4 \times 10^{-12} \pm 4.6 \times 10^{-13}$	9.4×10^{-12}
15.4 ± 2.1	70	$9.8 \times 10^{-13} \pm 2.3 \times 10^{-13}$	7.0×10^{-12}
19.1 ± 2.3	80	$7.3 \times 10^{-13} \pm 1.4 \times 10^{-13}$	6.13×10^{-12}
27.4 ± 3.6	150	$3.3 \times 10^{-13} \pm 8.2 \times 10^{-14}$	3.3×10^{-12}

This abrupt stop in the diffusion of 40 kDa PNIPMAM @ 33.4 ± 4.9 nm IONPs is perhaps due to some irreversible NP-gel fibre interactions (established slowly), which could be between NP core-gel fibre, polymer-gel fibre or both. To investigate it further, it was decided to check the effect of polymer chain length on the NPs diffusion, which would illustrate polymer-gel fibre interactions. The results are discussed in the following sections.

4.3.3. Effect of PNIPMAM chain length

Diffusion of different chain length PNIPMAM @ 15.4 ± 2.1 nm IONPs was studied through 0.5 % agar gel. The diffusion coefficient (**Fig. 4.7 (a, b)**) of PNIPMAM @ IONPs decreased with increase in PNIPMAM M_w from 7.5 kDa ($9.9 \times 10^{-13} \pm 1.9 \times 10^{-13} \text{ m}^2/\text{s}$) to 89 kDa ($1.06 \times 10^{-14} \pm 1.5 \times 10^{-15} \text{ m}^2/\text{s}$). Changes in PNIPMAM M_w led to only very small changes in D .



b)

Different M_w PNIPMAM @ 16 nm IONPs	D_{exp} (m^2 / s)
7.5 kDa	$9.9 \times 10^{-13} \pm 1.9 \times 10^{-13}$
40 kDa	$9.8 \times 10^{-13} \pm 2.3 \times 10^{-13}$
89 kDa	$1.06 \times 10^{-14} \pm 1.5 \times 10^{-15}$

Fig. 4.7. a) Collated $x(t)$ curves, and, **b)** experimentally determined diffusion coefficients ($R^2 > 0.98$) for the Brownian diffusion of 7.5, 40 and 89 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs through 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml. Hydrodynamic diameter (7.5, 40 and 89 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs) ~ 70 nm (**appendix F**), Theoretical $D = 7.0 \times 10^{-12} m^2/s$.

As the hydrodynamic diameter estimated by DLS wasn't reliable, polymer shell thickness calculations were used to estimate the diameter of a particle with a compact polymer layer of different M_w PNIPMAM @ 16 nm IONPs (**Table 4.2**). The actual diameter estimated for 89 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs was high (~ 22 nm) as compared to the 7.5 kDa PNIPMAM

@ 15.4 ± 2.1 nm IONPs (~ 18.3 nm). Theoretical D values were then calculated which were $2.7 \times 10^{-11} \text{ m}^2/\text{s}$ and $2.2 \times 10^{-11} \text{ m}^2/\text{s}$ for 7.5 kDa and 89 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs, respectively. This decrease in D_{theo} with increase in NP size was expected for a Fickian diffusion. However, there was only a small decrease in the experimental D (**Fig. 4.7 (b)**) for 7.5 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs ($9.9 \times 10^{-13} \pm 1.9 \times 10^{-13} \text{ m}^2/\text{s}$) and 89 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs ($1.06 \times 10^{-14} \pm 1.5 \times 10^{-15} \text{ m}^2/\text{s}$). This small change in D with the change in PNIPMAM M_w perhaps indicate no polymer-gel fiber interactions - or there may still be some weak interactions which are too weak to affect the diffusion trends.

Table 4.2. Estimated shell thickness and diameter of different M_w PNIPMAM @ 15.4 ± 2.1 nm IONPs.

Different M_w PNIPMAM @ 15.4 ± 2.1 nm (TEM) IONPs	Estimated diameter* of PNIPMAM @ IONP (nm)	Estimated PNIPMAM shell thickness on NP (nm)
7.5 kDa	~ 18.3	~ 1.4
19 kDa	~ 19.3	~ 1.9
27 kDa	~ 19.8	~ 2.2
40 kDa	~ 20.4	~ 2.5
89 kDa	~ 22.0	~ 3.3

Note. * See **appendix C** for calculations

The chain length of small ligand such as citric acid (CA) on NPs surface would be negligible compared to PNIPMAM and their diffusion would showcase the possible NPs core-gel interactions. Hence, the effect of the surface ligand on NP diffusion was explored where, the diffusion of small ligand (CA and tri-ethylene glycol (TREG)) coated NP was studied. Polymer-gel fibre interactions are further explored by studying the diffusion of hydrophilic poly-*N*-hydroxyethyl acrylamide (PNHEA) coated IONPs. The findings are reported below.

4.3.4. Effect of surface ligands on NPs diffusion

Coating IONPs with hydrophilic groups (e.g., hydroxyl and carboxylic acid groups) is essential for the formation of biocompatible aqueous suspensions. However, it is not unreasonable to suggest that these groups may possess high affinity for the gel functionalities. Hence, CA, TREG and PNHEA coated IONPs were studied for agar gel diffusion (**Fig. 4.8**). CA @ IONPs were obtained by the ligand exchange approach reported in the material and method chapter (chapter 6). TREG functionalized IONPs were prepared by a polyol synthesis. PNHEA was synthesized using RAFT polymerization (**appendix I, J**) and PNHEA @ IONPs were obtained using ligand exchange method similar to PNIPMAM.

Various size CA @ IONPs were studied for their diffusion behaviour through 0.5 % agar gel (**Fig. 4.9**). A size dependent diffusion similar to the core-shell NPs was observed (**Table 4.3**) where, CA @ 7.4 ± 1.4 nm IONPs diffused rapidly with the D_{exp} of $9.8 \times 10^{-12} \pm 5.6 \times 10^{-13} \text{ m}^2/\text{s}$ which was comparable to D_{theo} (4.08×10^{-11}). With further increase in NPs diameter, diffusion coefficient decreased to $3.5 \times 10^{-12} \pm 5.0 \times 10^{-13} \text{ m}^2/\text{s}$ for 19.1 ± 2.3 nm IONPs. The diffusion of 27.4 ± 3.6 nm IONPs was slowest and non-Fickian in nature, which indicates some weak NPs-gel fibre interaction or partial aggregation in the gel – or perhaps the NP were too big for the mesh size (~ 30 nm). Interestingly, CA @ 33.4 ± 4.9 nm IONPs didn't diffuse through the hydrogel which was in accordance with our hypothesis based on gel mesh size. But the size of 40 kDa PNIPMAM @ 33.4 ± 4.9 nm would be significantly high (~ 38 nm) as compared to mesh size, and hence their partial diffusion (**Fig. 4.6**) cannot be explained by this hypothesis.

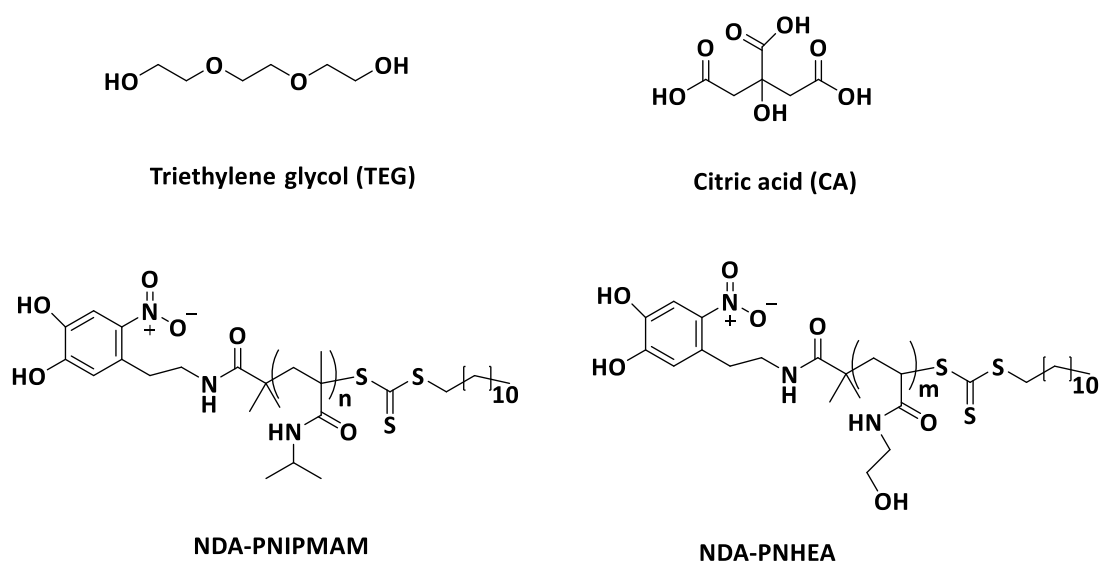


Fig. 4.8. Different ligands used to study the diffusion of coated IONPs.

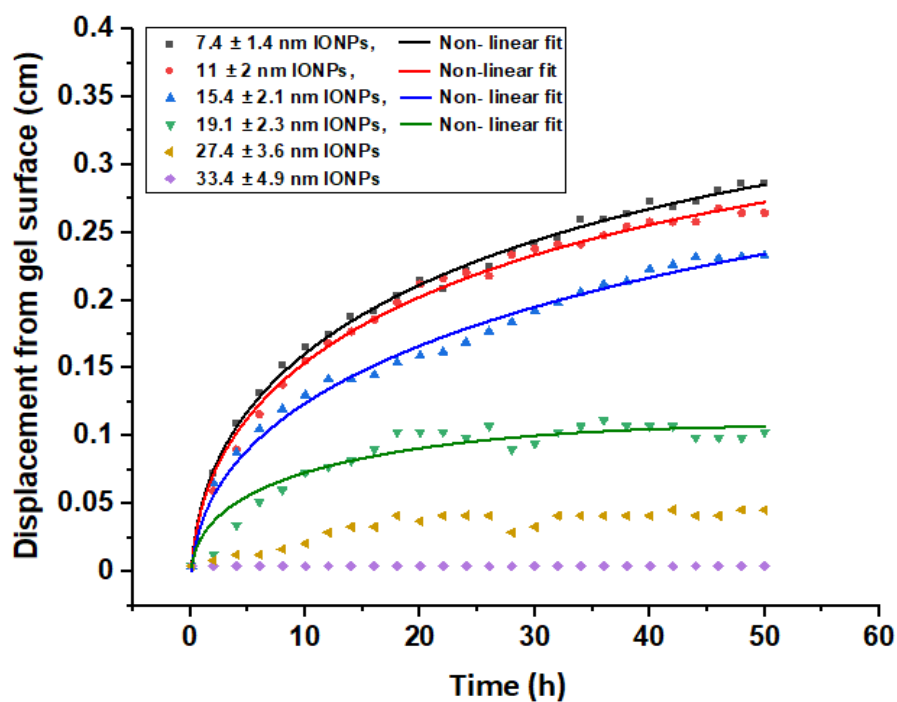


Fig. 4.9. Collated (t) curves with non-linear curve fitting ($R^2 > 0.94$) for the Brownian diffusion of various CA @ IONPs through 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml.

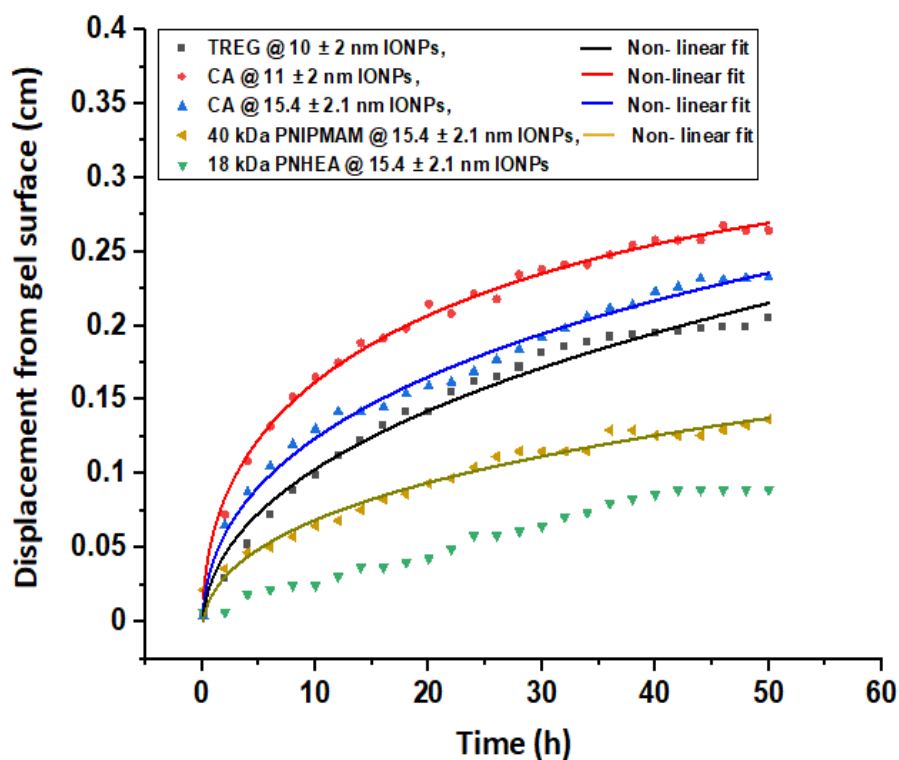
Table 4.3. Experimentally determined diffusion coefficients of CA @ 7, 11, 16 and 19 IONPs determined by non-linear curve fitting of the $x(t)$ curves for the Brownian diffusion. 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml. ($R^2 > 0.94$)

CA @ IONPs (nm, TEM)	Hydrodynamic diameter (nm, DLS)	D_{exp} (m^2 / s)	D_{theo} (m^2 / s)
7.3 ± 1.4	12	$9.8 \times 10^{-12} \pm 5.6 \times 10^{-13}$	4.08×10^{-11}
11 ± 2	13	$8.8 \times 10^{-12} \pm 7.8 \times 10^{-13}$	3.8×10^{-11}
15.4 ± 2.1	16	$5.0 \times 10^{-12} \pm 5.5 \times 10^{-13}$	3.06×10^{-11}
19.1 ± 2.3	30	$3.5 \times 10^{-12} \pm 5.0 \times 10^{-13}$	1.6×10^{-11}

In our DLS studies, the hydrodynamic diameter of CA @ 33.4 ± 4.9 nm IONPs was ~ 200 nm, which was significantly higher as compared to the core diameter. It is clearly indicating that the CA @ 33.4 ± 4.9 nm IONP were agglomerated, and hence didn't diffuse through the hydrogel. Polymer coating would solve this aggregation problem to some extent, which explains the initial diffusion of the 40 kDa PNIPMAM @ 33.4 ± 4.9 nm IONPs. In conclusion, CA @ IONPs diffusion through agar is Fickian and the NP-gel and ligand-gel interactions are minimal. For PNIPMAM @ IONPs, there is likely to be some interaction but still quite weak.

In gel-diffusion experiments through agar gel (**Fig. 4.10 (a, b)**), TREG coated IONPs (10 ± 2 nm) diffuse with a similar rate as compared ($D = 9.8 \times 10^{-12} \pm 5.6 \times 10^{-13} \text{ m}^2/\text{s}$) to the similar size and charge CA @ IONPs ($D = 8.8 \times 10^{-12} \pm 7.8 \times 10^{-13} \text{ m}^2/\text{s}$). These results showed that the diffusion of small ligand coated NPs is mainly governed by the hydrodynamic diameter (core size). Interestingly, the diffusion of the 19 kDa PNHEA @ 15.4 ± 2.1 nm IONPs was slower and non-Fickian suggesting some interactions between the gel fibres and the polymer functionalities.

a)



b)

TEM diameter	Hydrodynamic diameter (nm, DLS)	D_{exp} (m^2 / s)	D_{theo} (m^2 / s)
TREG @ 10 ± 2 nm IONPs	15	$9.8 \times 10^{-12} \pm 5.6 \times 10^{-13}$	3.3×10^{-11}
CA @ 11 ± 2 nm IONPs	13	$8.8 \times 10^{-12} \pm 7.8 \times 10^{-13}$	3.8×10^{-11}
CA @ 15.4 ± 2.1 nm IONPs	16	$5.0 \times 10^{-12} \pm 5.5 \times 10^{-13}$	3.06×10^{-11}
40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs	70	$9.8 \times 10^{-13} \pm 2.3 \times 10^{-13}$	7.0×10^{-12}

Fig. 4.10. a) Collated $x(t)$ curves and **b)** experimentally determined diffusion coefficients ($R^2 > 0.94$) for the Brownian diffusion of TREG, CA, PNIPMAM and PNHEA coated IONPs through 0.5 % agar gel (pH = 7). [NPs] = 5 mg/ml.

In conclusion, the factors determining NP diffusion are complex and depend strongly on both the ligand and the gelator material and can be difficult to rationalize. We further explored the effect of the charge in the gelator on NP diffusion by screening various IONPs through agarose gel. Since agarose is lacking anionic groups (agarpectin), the charge in the agarose hydrogel would be neutral and the NP diffusion would be compared to the negatively charged agar. The results are reported in the next section.

4.4. Diffusion of various size and surface ligand IONPs through agarose gel

Diffusion of different size PNIPMAM @ IONPs was studied through 0.5 wt% agarose hydrogel where there was no diffusion of core-shell NPs. It might possibly be due to NP-gel interactions which hindered the movement of polymer coated NP through agarose. The core-shell NP diffused partially through 0.1 wt% agarose (pH 7) where after some initial movement, diffusion of polymer coated NPs was ceased. Perhaps these NP-gel fibre interactions in agarose are slow but irreversible which stopped the NP movement through the gel over time. We then explored the effect of pH on the NP diffusion where unusual diffusion pattern for PNIPMAM @ IONPs was observed at high pH (pH 9). All PNIPMAM @ IONPs (7 to 27 nm) diffused (without stopping) through 0.1 % agarose gel (**Fig. 4.11**). Similar to agar, after the initial diffusion of the 40 kDa PNIPMAM @ 33.4 ± 4.9 nm IONPs, the movement of the NPs apparently stopped. Moreover, the effect of the hydrodynamic diameter on NP diffusion was similar to agar too but, their respective (t) curves were not in the agreement with the non-linear curve fitting. Hence, the diffusion of PNIPMAM @ IONPs through agarose gel at pH 9 was non-Fickian in nature, suggesting some polymer-gel interactions.

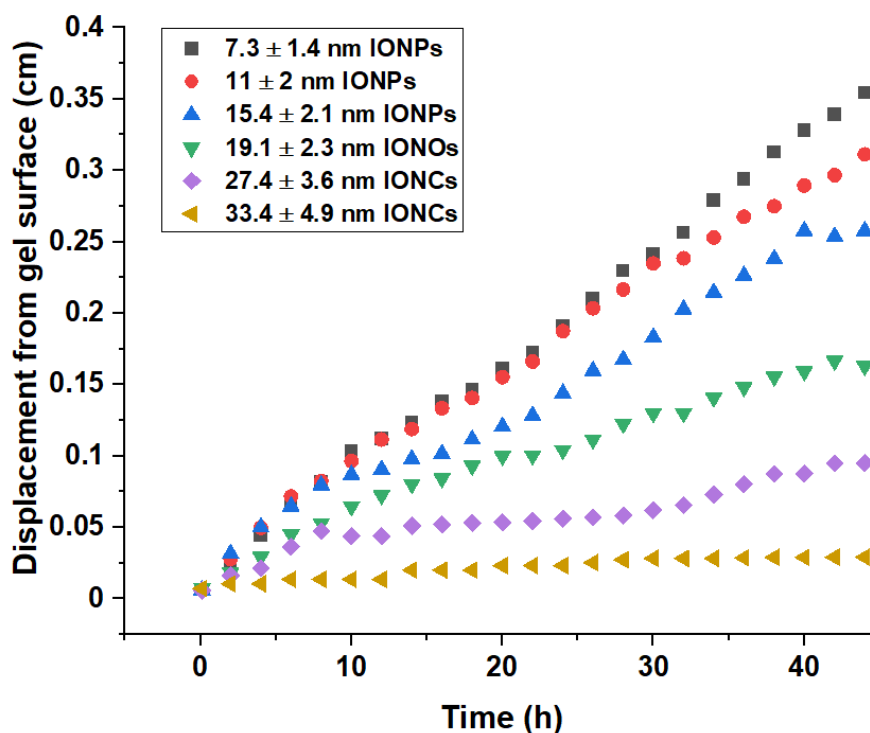
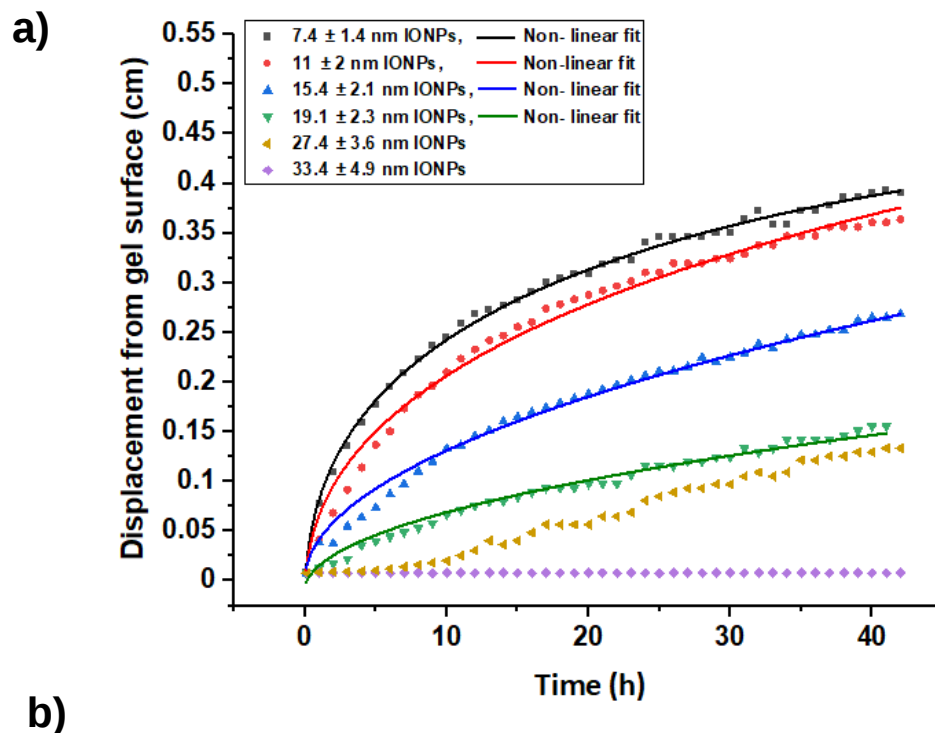


Fig. 4.11. Collated (t) curves of different size and shape PNIPMAM @ IONPs through 0.1 % agarose gel (pH 9). [NPs] = 5 mg/ml.

To investigate further on these polymer-gel interactions, CA @ IONPs were screened for the diffusion in 0.1 wt % agarose gel where NPs showed similar diffusion behavior at pH 7 and 9 (**Fig. 4.12 (b)**). These results suggest that the interactions between agarose and PNIPMAM @ IONP would be mainly polymer-gel fibre interaction.

Similar to agar, the diffusion of CA @ 7.3 ± 1.4 nm IONPs through agarose gel (pH 7) was quickest ($2.3 \times 10^{-11} \pm 1.0 \times 10^{-12} \text{ m}^2/\text{s}$) and no diffusion was observed for the NP above the 30 nm core diameter. Accurate measurement of the gel mesh size and NPs hydrodynamic diameter will give us more insight on this and hence could be an interesting future project.



CA @ IONPs (nm, TEM)	Hydrodynamic diameter (nm, DLS)	D_{exp} (m^2 / s , pH 7)	D_{exp} (m^2 / s , pH 9)	D_{theo} (m^2 / s)
7.3 ± 1.4	12	$2.3 \times 10^{-11} \pm 1.0 \times 10^{-12}$	$2.3 \times 10^{-11} \pm 1.3 \times 10^{-12}$	4.08×10^{-11}
11 ± 2	13	$1.0 \times 10^{-11} \pm 1.5 \times 10^{-12}$	$9.2 \times 10^{-12} \pm 1.7 \times 10^{-12}$	3.8×10^{-11}
15.4 ± 2.1	16	$6.12 \times 10^{-12} \pm 6.3 \times 10^{-13}$	$5.6 \times 10^{-12} \pm 1.0 \times 10^{-13}$	3.06×10^{-11}
19.1 ± 2.3	30	$3.9 \times 10^{-14} \pm 1.5 \times 10^{-15}$	$3.9 \times 10^{-14} \pm 2.2 \times 10^{-15}$	1.6×10^{-11}

Fig. 4.12. a) Collated $x(t)$ curves of various CA @ IONPs and b) experimentally determined diffusion coefficients ($R^2 > 0.94$) for the Brownian diffusion of 7, 11, 16 and 19 nm IONPs through 0.1 wt% agarose gel (pH = 7). [NPs] = 5 mg/ml.

Furthermore, PNHEA @ IONPs showed Fickian diffusion through agarose gel (0.1 wt%, pH 7) suggesting that the reason for slow diffusion of PNIPMAM IONPs in this gel are mainly polymer-gel interactions (**Fig. 4.13 (a, b)**). Moreover, the diffusion coefficient for CA @ 15.4 ±

2.1 nm IONPs ($6.12 \times 10^{-12} \pm 6.3 \times 10^{-13} \text{ m}^2/\text{s}$) was similar to 18 kDa PNHEA @ $15.4 \pm 2.1 \text{ nm}$ IONPs ($6.2 \times 10^{-12} \pm 8.14 \times 10^{-13} \text{ m}^2/\text{s}$). This was not obvious as you would expect polymer-coated NPs to diffuse slowly. However, because the surface coverage was low, the diameter of packed PNHEA @ IONPs ($\sim 17 \text{ nm}$) would not be much greater than that of CA @ IONPs ($\sim 16 \text{ nm}$). In any case, these are not straightforward arguments, they are quite speculative.

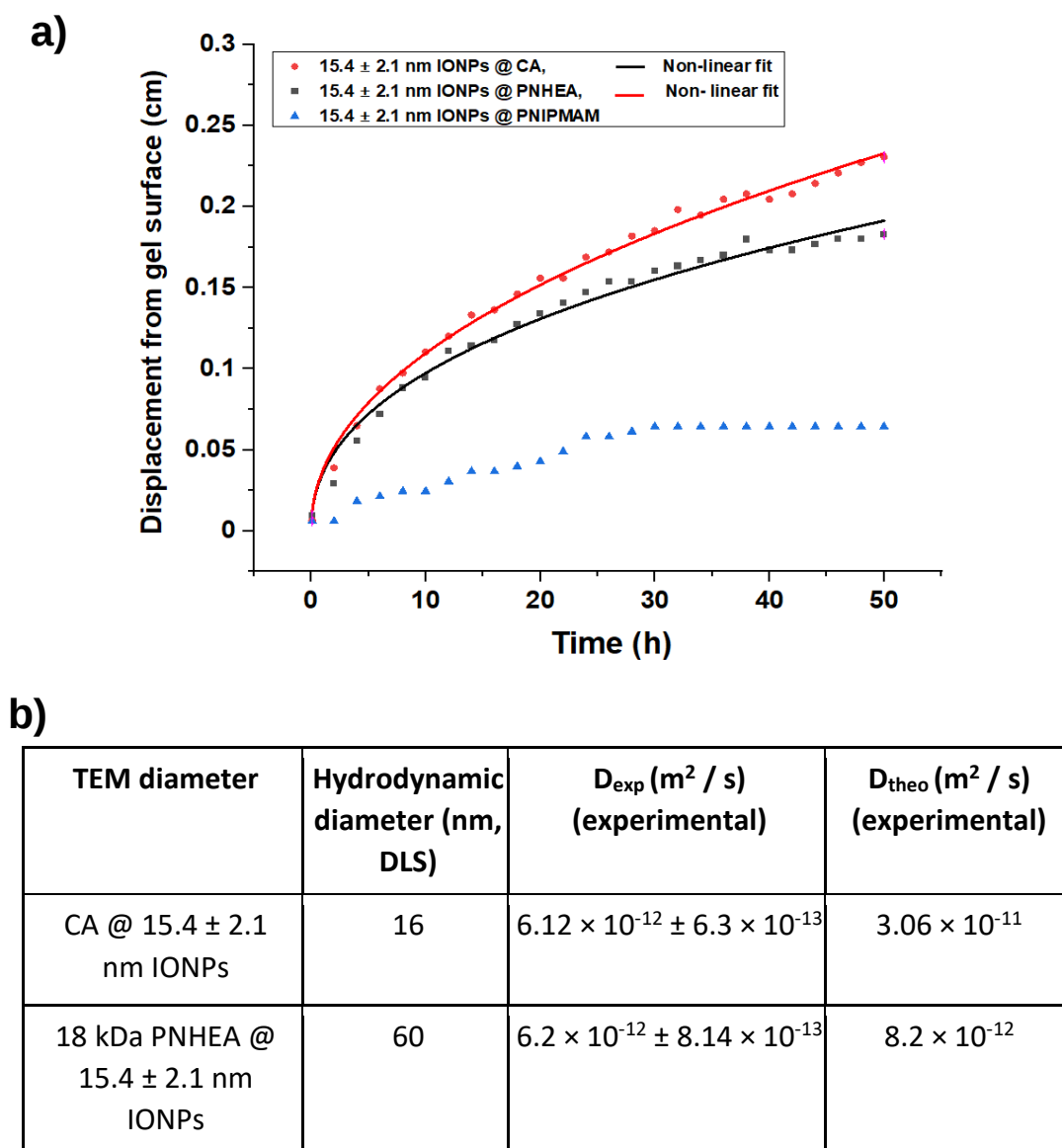


Fig. 4.13. a) Collated $x(t)$ curves and **b)** experimentally determined diffusion coefficients ($R^2 > 0.98$) for the Brownian diffusion of CA, PNIPMAM and PNHEA coated $15.4 \pm 2.1 \text{ nm}$ IONPs through 0.1 wt% agarose gel (pH = 7). [NPs] = 5 mg/ml.

Since the hydrogel composition was affecting the core-shell NPs diffusion, it was decided to check their diffusion through other hydrogels. The results are reported in the next section.

4.5. Effect of hydrogel functionalities on the diffusion of IONPs in gel model

Natural polymers-based hydrogels gelatin, pectin, agarose and carrageenan along with polyacrylamide and poly(ethylene glycol)diacrylate were studied for the diffusion of PNIPMAM @ IONPs (**Fig. 4.14**).

The main objective of these hydrogel screening experiments was to study the effect of gelator charge, H-bonding and hydrophobicity on NP diffusion. Interestingly, PNIPMAM @ IONPs diffused only through agar and agarose (pH = 9). Carrageenan contains sulphate functionalities similar to agar in its structure and will have an overall negative charge. Since agarose doesn't contain any sulphate groups, carrageenan result suggests that differences in diffusion cannot be explained by the presence of sulphate groups alone. In conclusion, the interactions between NP and gel fibres are complex and the interplay of hydrophobic, electrostatic and hydrogen bonding interactions which are difficult to unravel.

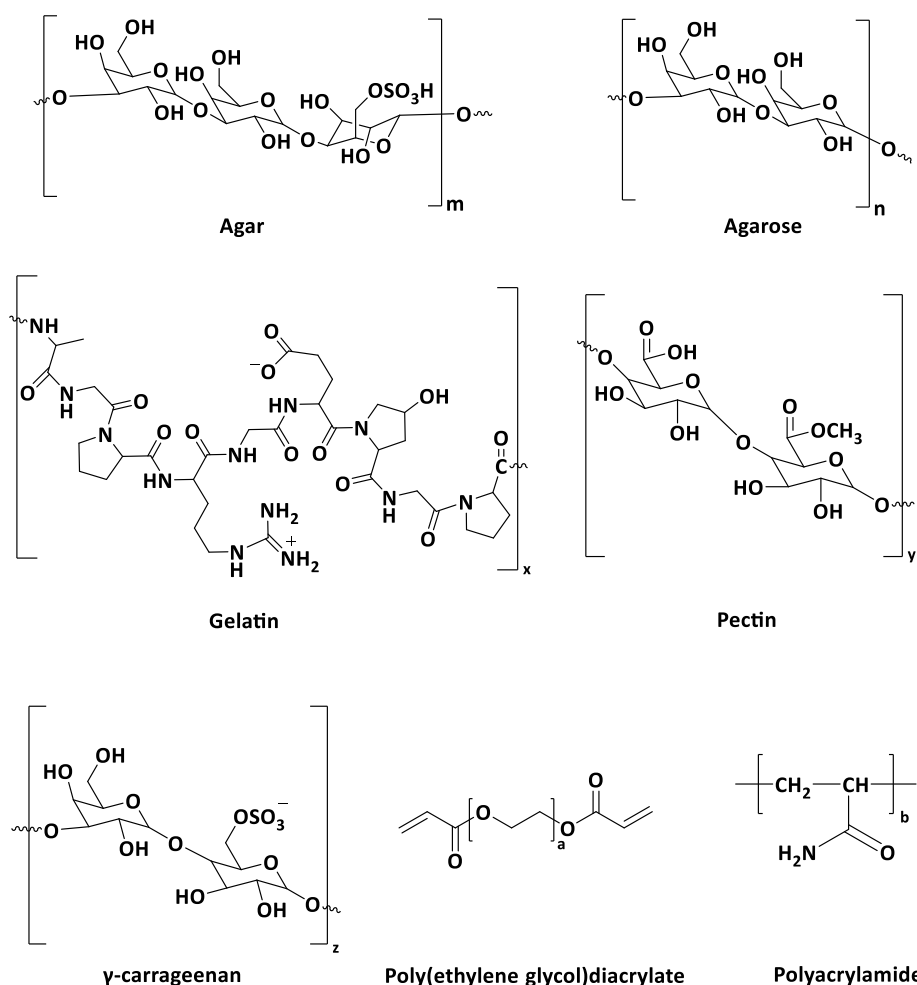


Fig. 4.14. Different hydrogels used to study the diffusion of PNIPMAM @ IONPs.

4.6. Conclusion

Agar gel was used for studying the diffusion of various IONPs. Diffusion of different size and surface ligands coated IONPs was monitored and diffusion coefficients were determined. Polymer coated NPs diffused slower through the gel as compared to citric acid coated IONPs. The key is that the coverage is very low and so a densely packed polymer shell would not increase NP diameter that much – this can explain small effect of the polymer chain length on diffusion. The diffusion of CA @ IONPs showed dependence on hydrodynamic diameter consistent with the Einstein-Stokes equation. NPs bigger than 30 nm (mesh size) couldn't diffuse through hydrogel suggesting a possible size limit on the NPs diffusion. However, for the polymer coated NPs (core size > 30 nm), the movement of the NPs stopped after some initial diffusion. This behavior suggests some slow but irreversible interactions of NP with the

gel fibres. Effect of functionalities on NPs surface was also studied where, the NP coated with a small ligand (TREG) showed similar diffusion behavior to the CA @ IONPs. For the hydroxyl-rich PNHEA-coated NPs, the diffusion became non-Fickian suggesting possible polymer-NPs interactions. In agarose hydrogel, PNIPMAM @ IONPs didn't diffuse through 0.5 wt% agarose hydrogel which illustrates some polymer-gel interactions. The core-shell NP diffused partially through 0.1 wt% (pH 7) agarose where the diffusion stopped showcasing the irreversible nature of NP-gel fibre interactions with agarose. At pH 9, there was an unusual diffusion of PNIPMAM @ IONPs through the agarose gel in non-Fickian manner. But the diffusion of the core-shell NP showed dependence on hydrodynamic diameter consistent with the Einstein-Stokes equation. These results illustrate the complexity of studying NP diffusion which are mainly governed by interactions with the surface ligands (H-bonding, hydrophobic and electrostatic interactions).

4.7. References

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Chapter 5: Protein encapsulation and triggered release from PNIPMAM @ IONPs

5.1. Introduction

Our group recently reported a successful encapsulation and triggered release of apotransferrin (TRF) protein from poly-*N*-isopropylacrylamide (PNIPAM) coated IONPs⁷ (**Table 5.1**). Poly-*N*-isopropylmethacrylamide (PNIPMAM) only differs from PNIPAM by one methyl group in the repeat unit, it could be expected to display similar protein encapsulation/triggered release behaviour.

Table 5.1. Comparison of molecular weight and isoelectric point of TRF and RNaseB.

Protein	Molecular weight (kDa)	Isoelectric point (PI)	Charge on protein at pH 7.4
RNaseB	14.7	9.3	Positive
TRF	80	5.4	Negative

Understanding protein-NP interaction is critical for optimising encapsulation and release processes.¹⁻⁷ In our case, the polymer grafting density on the NP surface was low (0.03 chains/nm², TGA) and hence, the polymer shell thickness on the NP surface would be low (~ 2 nm for 40 kDa PNIPMAM coated NPs). Therefore, it was expected that the protein would interact with both iron oxide core and the polymer shell on core-shell NP (**Fig. 5.1**).

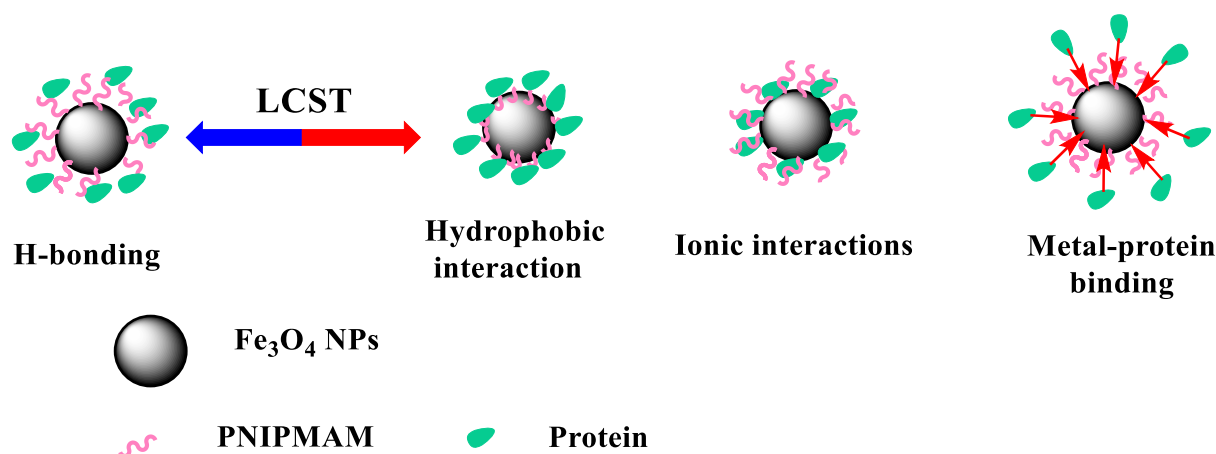


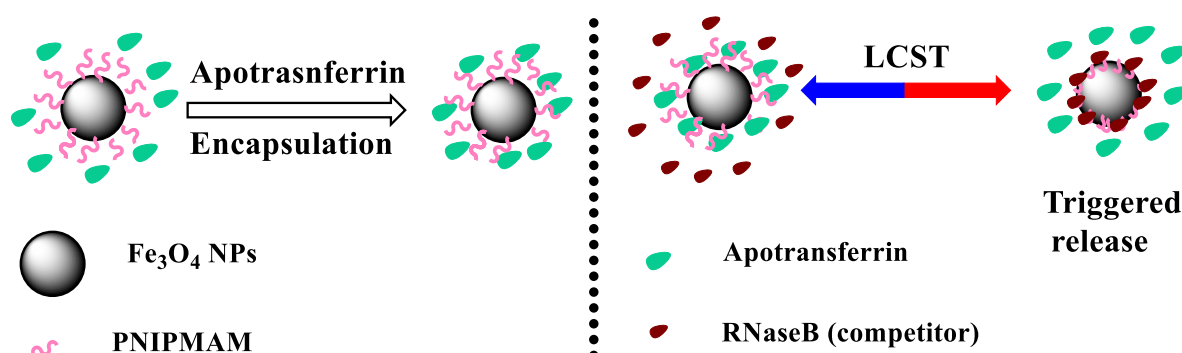
Fig. 5.1. Various modes of protein-NP interactions.

In this paragraph I will consider the main types of these NP-protein interactions. Proteins are biological amphoteric molecules contain both acidic and basic functional groups. Amino acids that make up proteins may be positive, negative or neutral in nature, and together give a protein its overall charge. Isoelectric point (PI) is the pH at which a protein carries a net zero charge. Consequently, proteins exhibit negative charge above and positive charge below their PI (**Table 5.1**). Zeta potential measurements of our core-shell NPs (PNIPMAM @ IONPs) revealed an overall negative surface charge (~ -3 mV, DLS) on the NP. There are no ionizable groups in the PNIPMAM repeat units (pH 7.4) and hence this negative charge would be on the IONP core. Since both proteins and core-shell NP have an overall charge (at physiological pH), the interactions between protein-NP could be electrostatic in nature. Additionally, the amino acids (mainly histidine or cysteine) in the protein structure are known to bind to the metal ions (Ca^{2+} , Fe^{2+} , Zn^{2+} etc.), which in some cases is a necessary part of their folding and maintenance of a tertiary structure.¹⁰ There are some reports where researchers utilized this metal-protein binding to develop artificial metalloenzymes.¹¹ Hence, the amino acid functionalities of protein could bind to the NP core (containing iron oxide) which would aid in the protein encapsulation. Moreover, the amide functionalities in PNIPMAM repeat units can encapsulate protein by the H-bonding. Below the lower critical solution temperature (LCST) of the core-shell NP, the polymer chains on the NP surface would be in the expanded form, which makes the polymer functionalities more accessible for the protein encapsulation. However, above the LCST, the polymer chains collapse on the NP surface, which would essentially

resemble a big hydrophobic sphere. Hence, the protein-NP interactions above the LCST of PNIPMAM @ IONPs are proposed to be mainly hydrophobic interactions. In conclusion, protein encapsulation to PNIPMAM @ IONPs would be a combined effect of the electrostatic interactions and metal ion-binding of the protein with the NP core and, H-bonding (below the LCST) and hydrophobic interactions (above the LCST) of the protein with the polymer shell.

However, it is difficult to comment on the arrangement of polymer chains on the NPs core¹⁷ and hence core accessibility for the NP-protein interactions. Bigger NPs coated with smaller polymers could experience depletion forces pushing the particles together.¹⁸ It would make them agglomerated and less available for the protein encapsulation. However, polymers bigger than NPs would expand and wrap around the core making them more stable. With further increase in chain length, the polymer shell around the core would become thicker. It would make the accessibility of the protein molecules to the NPs core difficult. Additionally, there would be plenty of polymer chains for the protein to interact which would increase the protein encapsulation. Therefore, it was decided to study the size effect of NP core, along with the polymer chain length on TRF encapsulation/release.

In order to achieve that, PNIPMAM @ IONPs were subjected to TRF encapsulation and triggered release in the presence of RNaseB (**Scheme 1**). For mimicking high protein concentrations in the biological media (serum), excess competitor protein (RNaseB, 10 mg/ml) was used for the TRF release experiments.⁷



Scheme 5.1. TRF encapsulation to PNIPMAM @ IONPs and its triggered release above LCST (45 °C) in the presence of a competitor protein RNaseB.

In the next section, the optimization of the core-shell NPs for the TRF encapsulation/triggered release are discussed. After obtaining an optimized core-shell structure, effect of protein size (molecular weight), charge (PI) and glycosylation (discussed in section 5.3) on their encapsulation/triggered release are discussed. Additionally, serum was also discussed as a potential source of competitor proteins for *in vivo* applications.

5.2. Optimization of core-shell NPs

5.2.1. Effect of polymer chain length on TRF release

Phase transition of the polymer was utilized to encapsulate protein to PNIPMAM@IONPs. This was conducted by incubating a protein containing PNIPMAM @ IONPs suspension above the LCST (45 °C) before gradually cooling to room temperature. PNIPMAM @ IONPs would collapse above the LCST and the proteins may (weakly) bind to the NP surface. With the subsequent cooling to room temperature, polymer would re-expand around the proteins in the solution, resulting in encapsulation. After encapsulation, triggered release of TRF in the presence of RNaseB was studied. Western blotting was used to quantify the protein loading and release (**appendix K-M**).

15.4 ± 2.1 nm (average diameter ± standard error) IONPs were selected as common core material to study the effect of the molecular weight of PNIPMAM on protein encapsulation and release. TRF loading was increased with increase in PNIPMAM molecular weight on the NPs surface. For 0.5 mg of PNIPMAM @ IONPs ([Fe]), there was little protein loading (~ 5 % of 1000 ng (50 ng)) for 7.5 kDa PNIPMAM which increased to ~ 40 % (400 ng) for 40 kDa polymer. With further increase in molecular weight to 89 kDa, protein loading was increased to ~ 70 % (700 ng, **Fig. 5.2**).

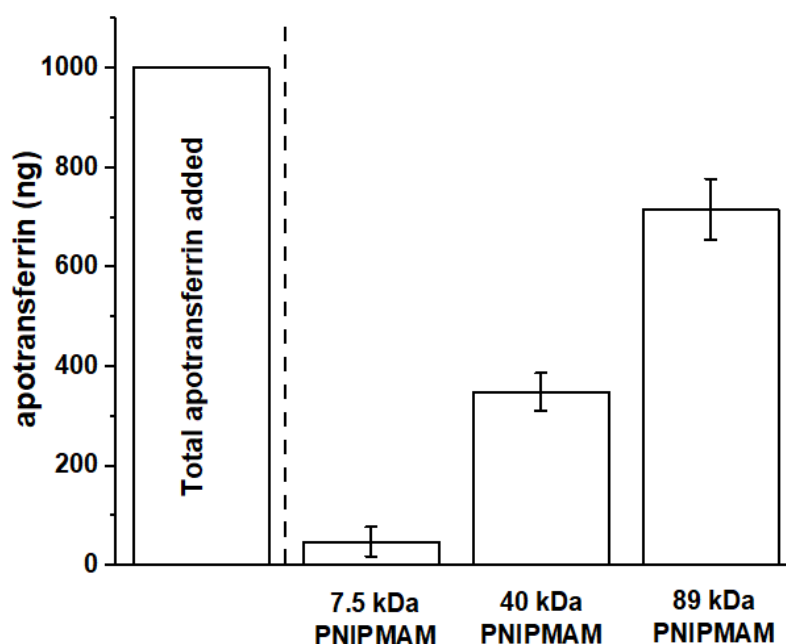


Fig. 5.2. Effect of polymer chain length on TRF loading: 0.5 mg of 15.4 ± 2.1 nm IONPs coated with different molecular weight PNIPMAM (7.5, 40 and 89 kDa) were incubated with 1 μ g TRF followed by separation of NPs using centrifugation. Western blot analysis (**appendix K**) and quantification of the supernatant was done to quantify unloaded TRF ($n = 3$, error bars denote standard error).

Increase in protein loading with increase in molecular weight of PNIPMAM was an indirect evidence of possible interactions between proteins and polymer chains. After protein encapsulation, its triggered release was studied at 45 °C (heating experiment) in the presence of RNaseB. Control experiment at 37 °C was run parallel to the heating experiment to verify that release above the LCST was indeed triggered by the polymer phase transition. No TRF release was observed in the absence of competitor protein which was in accordance with our previous results.⁷

With the different chain length PNIPMAM @ IONPs, no protein release was observed for 7.5 kDa and 19 kDa polymer coated NPs (**Fig. 5.3**). For 27 kDa PNIPMAM, small amount of protein release was observed (~ 1 ng, 45 °C) accompanied by similar levels of protein leaching at 37 °C. There was an improvement in the triggered release at 45 °C (~ 20 ng) for 40 kDa PNIPMAM with minimum leakage (~ 1 ng) at 37 °C. With further increase in molecular weight to 89 kDa,

there was 2-fold increase in triggered protein release (~ 40 ng). However, TRF leaching at 37 °C was also increased (~ 15 ng).

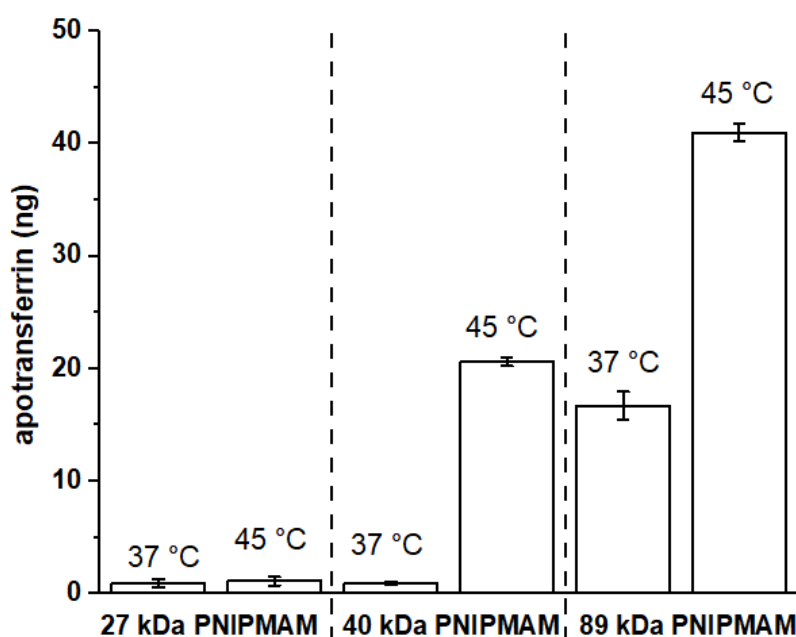


Fig. 5.3. Effect of polymer chain length on triggered TRF release in a temperature-dependent manner: 0.5 mg of 15.4 ± 2.1 nm IONPs coated with different molecular weight PNIPMAM (7.5, 19, 27, 40 and 89 kDa) were incubated with 1 μ g TRF followed by separation of NPs using centrifugation. Western blot analysis (**appendix K**) and quantification of TRF release (with 10 mg/ml RNase B) from PNIPMAM @ IONPs (27, 40 and 89 kDa) at 37 °C and 45 °C (for 1 h), respectively ($n = 3$, error bars denote standard error). Protein release was calculated relative to the amount of TRF associated with the particles after 10 washings.

The protein leak at 37 °C could be due to loosely bound protein to PNIPMAM @ IONPs. In addition to TRF encapsulation, this loosely bound protein was indicating the complexity of protein binding to the polymer coated NPs. To address this issue, prolonged washings with the competitor protein were performed (10 washes, each with 4h of incubation at 37 °C). Longer washing time was found more effective in removing loosely bound protein than increasing number of washes. Since the protein leak was significantly lower for 40 kDa PNIPMAM than 89 kDa PNIPMAM, it was decided to use 40 kDa PNIPMAM for further investigations on protein-NP interactions.

The experimental findings for the size optimization of IONPs for TRF encapsulation/release are described in the next section.

5.2.2. Effect of IONPs size on TRF encapsulation and release

For studying the effect of the NPs core diameter on protein encapsulation and release, 7.3 ± 1.4 nm, 11 ± 2 nm and 15.4 ± 2.1 nm IONPs were investigated. 40 kDa PNIPMAM was used as a common polymer shell on the NPs surface and the same amount of NPs (based on the mass of Fe) was used in these experiments. TRF loading decreased with increase in NPs diameter where maximum loading was observed for 7.3 ± 1.4 nm IONPs ($\sim 90\%$ of 1000 ng TRF, **Fig. 5.4**). It was expected due to the high surface area of smaller 7.3 ± 1.4 nm IONPs. More surface area would result in more PNIPMAM chains on the NPs surface and hence more protein loading. After TRF loading, control and heating experiments were done on the protein loaded NPs and Western blot analysis was performed (**appendix L**).

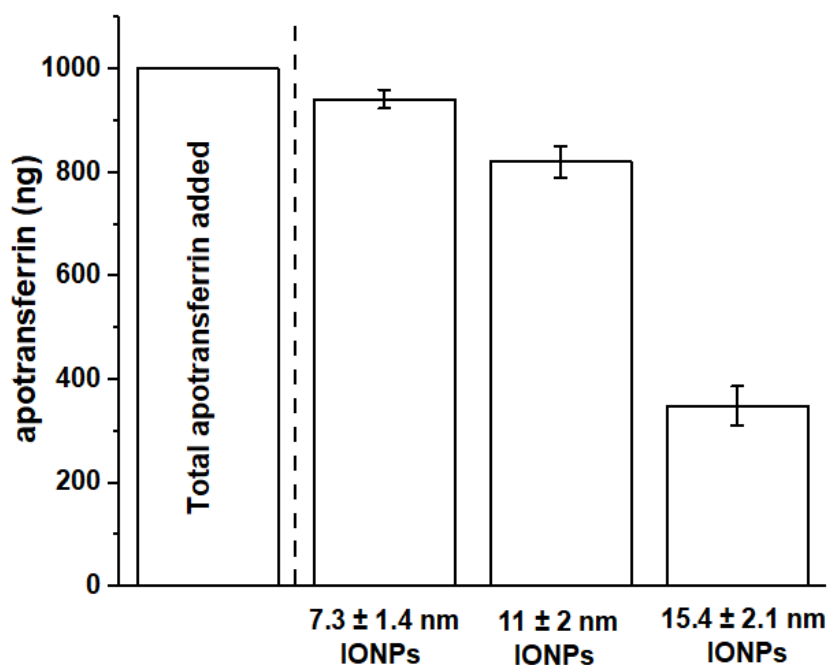


Fig. 5.4. Effect of IONPs size on TRF loading: 0.5 mg of different size IONPs (7, 11 and 16 nm) coated with 40 kDa PNIPMAM were incubated with $1\mu\text{g}$ TRF followed by separation of NPs

using centrifugation. Western blot analysis (**appendix L**) and quantification of the supernatant was done to quantify unloaded TRF ($n = 3$, error bars denote standard error).

Interestingly, different core IONPs showed different protein release behaviour (**Fig. 5.5**). Despite higher protein encapsulation, lower amount of TRF was released from 7.3 ± 1.4 nm (~ 7 ng) and 11 ± 2 nm IONPs (~ 5 ng). However, 15.4 ± 2.1 nm IONPs were performing well with a reasonable triggered protein release (~ 21 ng) at 45°C and a slight protein leak (~ 1 ng) at 37°C (**Fig. 5.5**). The smaller particles have higher curvature and hence greater potential for the protein to irreversibly bind to Fe oxide, which could be the reason for lower protein release.

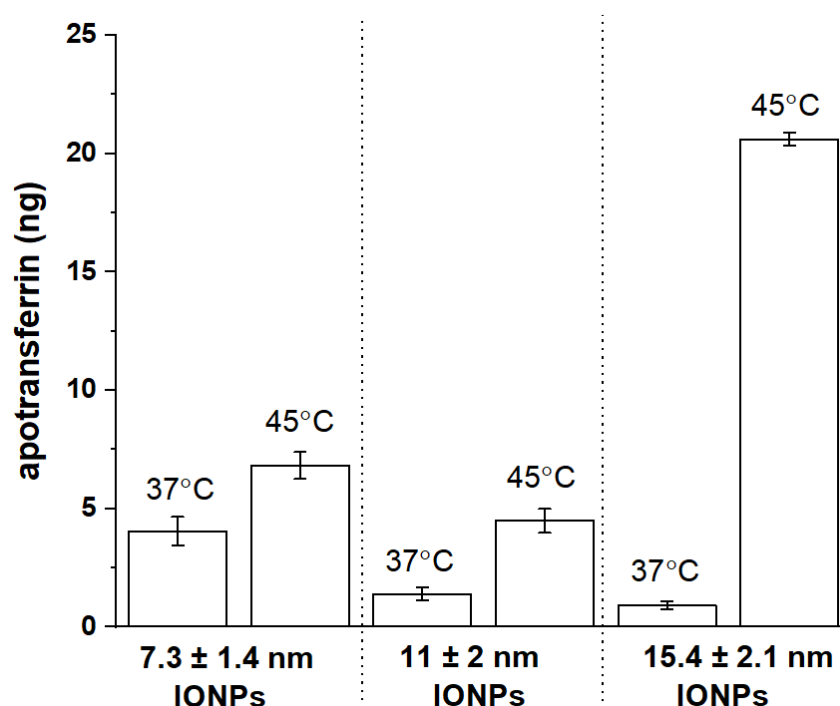


Fig. 5.5. Effect of IONPs size on triggered TRF release in a temperature-dependent manner: 0.5 mg PNIPMAM @ IONPs (40 PNIPMAM @ 7, 11 and 16 nm IONPs) were incubated with $1 \mu\text{g}$ TRF followed by separation of NPs using centrifugation. Western blot analysis (**appendix L**) and quantification following 10 mg/ml competitor protein treatment (RNaseB) for TRF release from PNIPMAM @ IONPs at 37°C and 45°C (for 1 h), respectively ($n = 3$, error bars denote standard error). Protein release was calculated relative to the amount of TRF associated with the particles after 10 washings.

In conclusion, protein encapsulation increased with increase in polymer chain length and decrease in NP core size where, maximum protein loading was obtained for 40 kDa @ 7 nm IONPs. However, the protein release profile was not satisfactory for 7 nm IONPs and hence, it was decided to use 40 kDa PNIPMAM @ 16 nm IONPs for further experiments.

In the next section, results on the effect of protein properties mainly size, PI and glycosylation on their encapsulation and triggered release from 40 kDa PNIPMAM @ 16 nm IONPs are discussed.

5.3. Effect of protein properties on their encapsulation and triggered release

Protein-NP interaction mainly depends on the physicochemical properties of both NP and protein (size, charge, surface functional groups, shape). Bigger proteins will have more residues available for interaction with the NP. Perhaps bigger competitor than RNaseB might release more TRF from PNIPMAM @ IONPs. Additionally, due to small negative charge on PNIPMAM @ IONPs, PI can also play some role in protein encapsulation/release. Our gel diffusion experiments also showcased the possibility of NP-polysaccharide interactions to be electrostatic. But if we assume ionic bonding between TRF and the coated NPs, the oppositely charged RNaseB would not bind to the same sites on the coated NP, and would not release the TRF by screening ionic interactions. The triggered TRF release with RNaseB suggested no direct effect of PI on protein encapsulation/release. However, to consider this hypothesis, screening other positive proteins for the trigger TRF release is required.

Protein glycosylation is the most common form of post-translational modification (PTM) on excreted and extracellular membrane-associated proteins. It involves the covalent attachment of many different types of glycans to a protein. Glycosylated proteins contain covalently attached carbohydrates in their structure (**Fig. 5.6**), and this modification serves various functions such as improving protein folding and stability. There are some reports suggesting the role of glycosylation on the thermodynamic and kinetic stability of proteins.¹² Glycosylation affects the layout of binding site residues and transferrin structure.¹³ As

glycosylation can modify protein shape and orientation of surface functionalities, it could have some effect on protein-NP interactions.

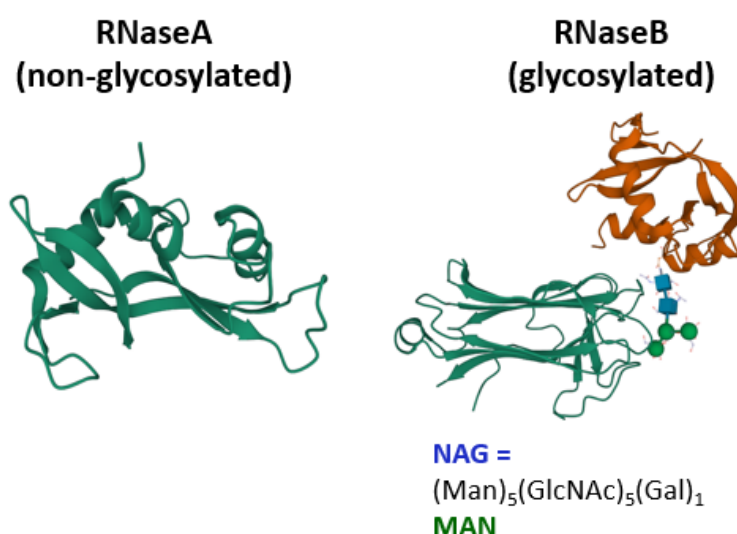


Fig. 5.6. Structure differences between similar size and PI (9.3) non-glycosylated RNaseA (13.9 kDa, PDB = 7RSA) and glycosylated mannose rich RNaseB (14.9 kDa, PDB = 2E33). Here, NAG (GlcNAc) = *N*-acetylglucosamine, MAN = mannose and Gal = galactose.

In order to determine the main protein properties (size, PI and glycosylation) affecting protein-NP interactions, it was decided to screen different competitor proteins for the triggered TRF release. But due to high concentration requirement (10 mg/ml) of the competitor in release experiments, the competitor protein options were limited and hence economically viable options were explored. Ovalbumin (OVL, ~ 45 kDa, PI = 5.2) and bovine immunoglobulin (IgG, ~ 160 kDa, PI = 7.3) were selected as glycosylated and RNaseA (~ 13.7 kDa, PI = 9.3) and bovine serum albumin (BSA, ~ 66 kDa, PI = 4.5) were selected as non-glycosylated competitors. This including RNaseB (~ 14.7 kDa, PI = 9.3) was a good competitor protein range for studying the effect of size and PI on triggered protein release.

In the following section, experimental findings for the triggered TRF release with different size and PI glycosylated competitors are reported. Subsequently, the results on the TRF release with non-glycosylated proteins are discussed.

5.3.1. TRF release with different competitor proteins (glycosylated)

Following TRF encapsulation to 40 kDa PNIPMAM @ 16 nm IONPs, release experiments with different glycosylated competitor proteins were performed (**Table 5.2**).

Table. 5.2. Comparison of molecular weight and isoelectric point of TRF, RNaseB, ovalbumin and IgG.

Glycoproteins used	Molecular weight (kDa)	Isoelectric point	Charge on protein at pH 7.4
TRF	80	5.4	Negative
RNaseB	14.7	9.3	Positive
OVL	45	5.2	Negative
IgG	160	7.3	Neutral

As expected, with increase in competitor size from 14.9 kDa (RNaseB) to 45 kDa (OVL), amount of TRF release (45 °C) was increased from ~ 10 % to ~ 50 % of the loaded protein (~ 400 ng) (**Fig. 5.7**). However, some elevation in protein leak (37 °C) from ~ 0.3 % (RNaseB) to ~ 2 % (OVL) was also observed. There was a further increase in the protein release with IgG (~ 160 kDa) to ~ 90 % (~ 350 ng, 45 °C) accompanied by higher protein leak (~ 100 ng, 37 °C). Higher M_w proteins would interact with NPs stronger which results in higher release numbers.

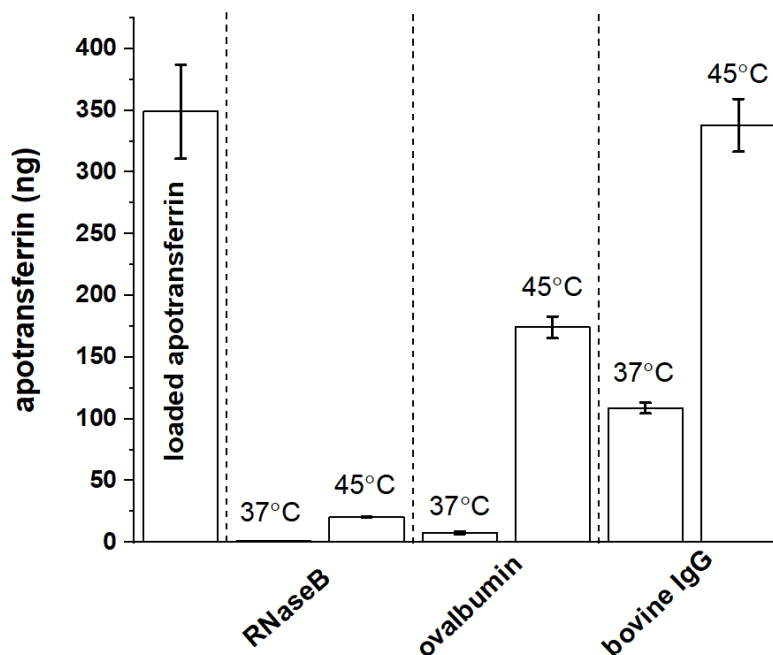


Fig. 5.7. PNIPMAM @ IONPs release TRF in a temperature-dependent manner with different competitor proteins: 0.5 mg 40 kDa PNIPMAM @16 nm IONPs were incubated with 1 μ g TRF followed by separation of NPs using centrifugation. Western blot analysis (**appendix L, M**) and quantification 10 mg/ml competitor protein treatment (RNaseB, OVL and IgG) for the TRF release from PNIPMAM @ IONPs at 37 °C and 45 °C (for 1 h), respectively (n = 3, error bars denote standard error). Protein release was calculated relative to the amount of TRF associated with the particles after 10 washings.

In the next section, results on triggered TRF release with different non-glycosylated competitor proteins are reported.

5.3.2. Effect of protein glycosylation on triggered release

Because of similar molecular weight and PI to the glycosylated RNaseB, non-glycosylated RNaseA was an ideal candidate for studying the effect of protein glycosylation on triggered protein release (**Table. 5.3**).

Table. 5.3. Different competitor proteins used to study the effect of protein size, isoelectric point and glycosylation on the triggered release of TRF from PNIPMAM @ IONPs.

Competitor proteins used	Molecular weight (kDa)	Isoelectric point	Charge on protein at pH 7.4
RNaseA*	14.7	9.3	Positive
RNaseB	14.7	9.3	Positive
OVL	45	5.2	Negative
BSA*	66	4.5	Negative
IgG	160	7.3	Neutral

Note. * = non-glycosylated protein

Following TRF encapsulation to PNIPMAM @ IONPs, release experiments were performed with RNaseA as a competitor. Interestingly, triggered TRF release levels (**Fig. 5.8**) were significantly lower (~ 2 ng) as compared to glycosylated RNaseB (~ 20 ng). Perhaps glycans would have helped RNaseB bind better to the NPs surface to release bound TRF.

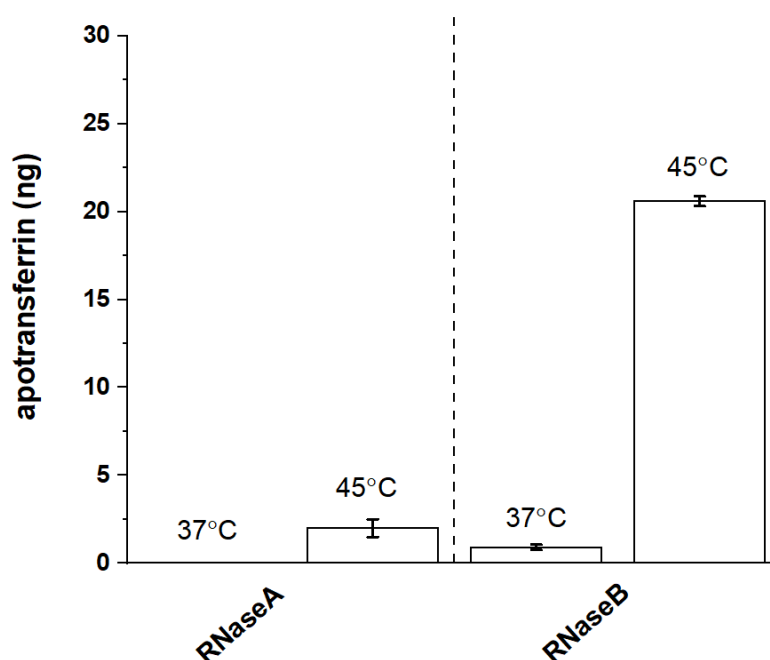


Fig. 5.8. Effect of protein glycosylation on triggered release of TRF from PNIPMAM @ IONPs in a temperature-dependent manner: 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs were incubated with 1 µg TRF followed by separation of NPs using centrifugation. Western blot

analysis (**appendix L, M**) and quantification following 10 mg/ml competitor protein treatment (RNaseA and RNaseB) for the TRF release from PNIPMAM @ IONPs at 37 °C and 45 °C (for 1 h), respectively (n = 3, error bars denote standard error). Protein release was calculated relative to the amount of TRF associated with the particles after 10 washings.

To investigate it further, a larger non-glycosylated protein bovine serum albumin (BSA, 66 kDa) was screened as a competitor protein (**Fig. 5.9**). Since it has bigger size than OVL (45 kDa), higher TRF release was expected. However, the amount of protein release with BSA (~ 30 ng) was significantly less than OVL (~ 150 ng). This is consistent with the previously observed effect of protein glycosylation in triggered protein release.

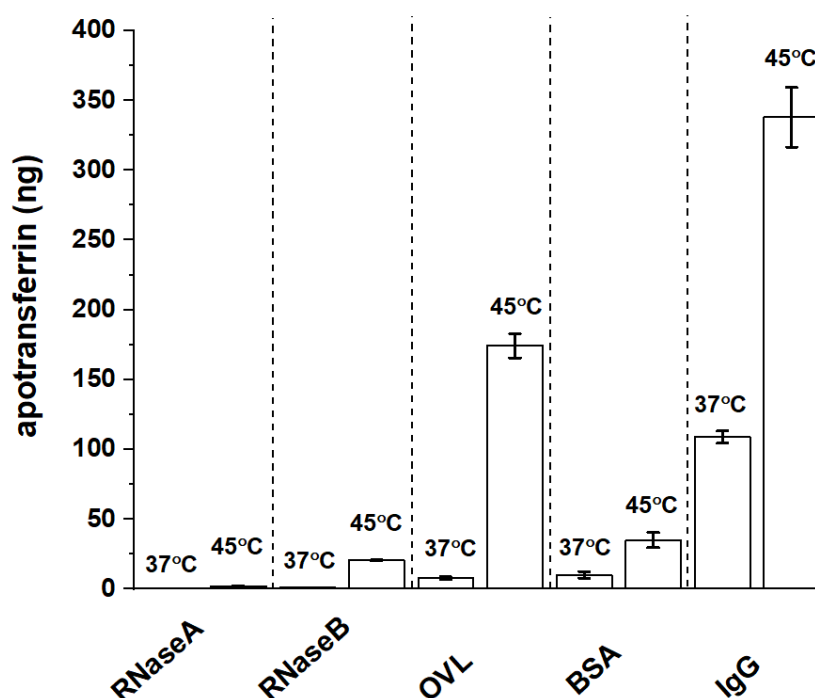


Fig. 5.9. PNIPMAM @ IONPs release TRF in a temperature-dependent manner with different competitor proteins: 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs were incubated with 1 µg TRF followed by separation of NPs using centrifugation. Western blot analysis (**appendix L, M**) and quantification following 10 mg/ml competitor protein treatment (RNaseA, RNaseB, OVL, BSA and IgG) for the TRF release from PNIPMAM @ IONPs at 37 °C and 45 °C (for 1 h), respectively (n = 3, error bars denote standard error). Protein release was calculated relative to the amount of TRF associated with the particles after 10 washings.

In conclusion, the presence of glycan on the protein surface appears to facilitate release. To explore this further, it was decided to check different carbohydrates as competitors for the triggered TRF release. The main objective of these experiments was to explore the possibility of sugar-NP interactions in glycosylated proteins. The results are reported in the next section.

5.4. Sugars as competitors in triggered TRF release from PNIPMAM @ IONPs

Glycans can be homo- or heteropolymers of monosaccharide residues (mainly mannose, galactose, glucose, fucose etc.), and can be linear or branched. Hence three monosaccharides (D-mannose, D-galactose and D-glucose) along with a disaccharide (maltose) were screened as competitors for the triggered TRF release (**Fig. 5.10**). As glycan attached to a protein would have a rather complex structure, these sugars weren't a best glycan mimic. Therefore, a polysaccharide (alginic acid) was also screened as a competitor for the TRF release. Its molar mass could be between 10kDa to 600kDa and hence would have bigger size than most of the competitor proteins used in this study (**Fig. 5.10**). Additionally, the presence of carboxylate groups in alginic acid would provide information on whether binding to the NP core is an important factor for the release.

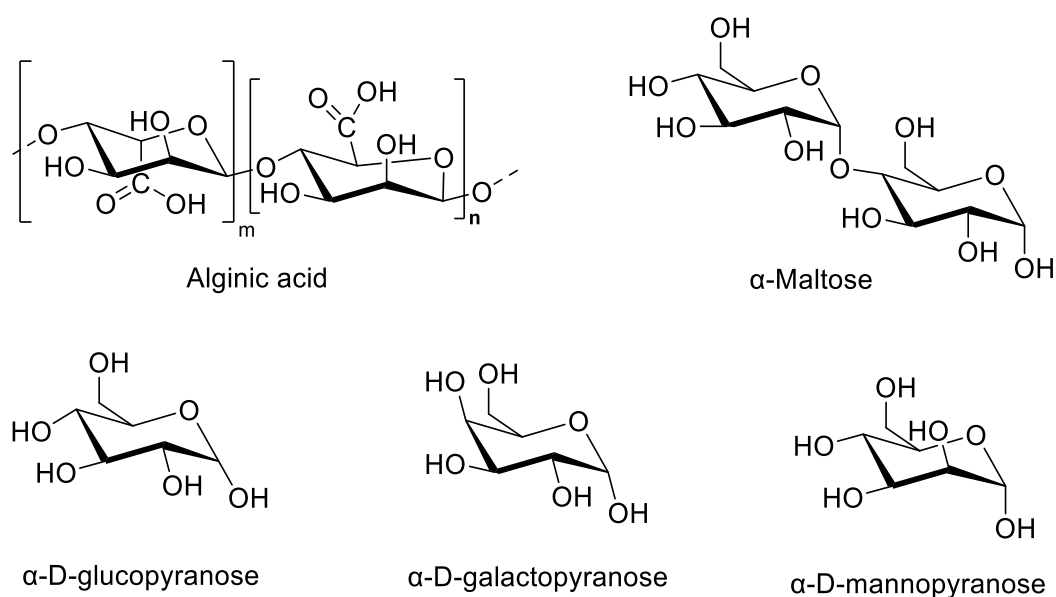
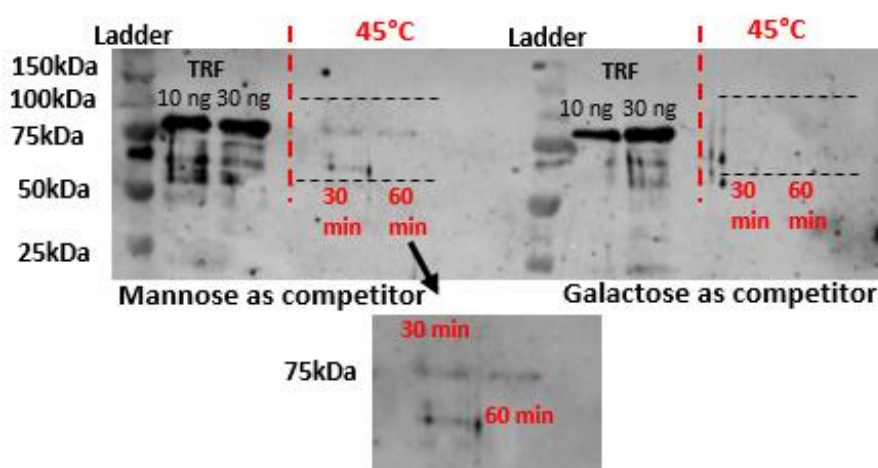


Fig. 5.10. Various saccharides used in this study as competitors (10 mg/ml, pH = 7.4) for triggered protein release from PNIPMAM @ IONPs: An acidic polysaccharide alginic acid, a glucose disaccharide maltose, D-glucose, D-galactose and D-mannose.

Protein encapsulated NPs were treated with above mentioned saccharides (10 mg/ml, pH 7.4) above the LCST (45 °C). Interestingly, only D-mannose resulted in a partial release of TRF above the LCST of PNIPMAM @ IONPs (**Fig. 5.11 (a, b)**). These results indicate no binding of saccharides (except mannose) with high enough strength to the TRF binding sites in the coated NP to cause TRF release. Perhaps they may even bind to the TRF binding sites but too weakly to compete with TRF.

These selective interactions of mannose with polymer coated IONPs were compelling. Meng *et. al.* also found mannose as a better chelator for iron as compared to glucose and other sugars.¹⁴ Additionally, the glycoproteins used in this study are mannose rich.^{15,16} Perhaps these selective interactions with mannose could be the reason for higher activity of glycosylated proteins as competitor. If we consider this hypothesis, then mannose rich glycoproteins would be better competitors than non-glycosylated proteins.

a)



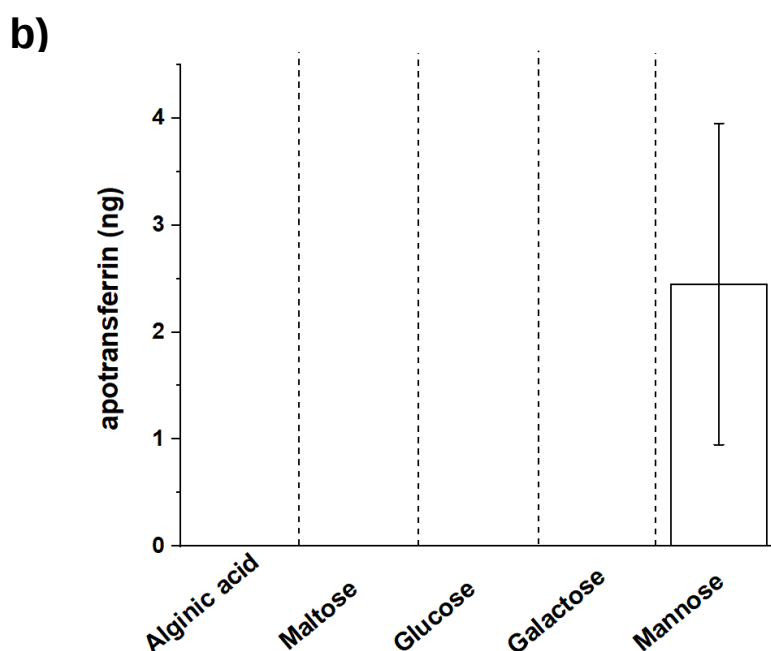


Fig. 5.11. Triggered release of TRF from PNIPMAM @ IONPs in a temperature-dependent manner with different sugars as competitor: a.) Western blot analysis of mannose and galactose as a competitor on a same blot for comparison following mannose and galactose treatment (10 mg/ml) of 0.5 mg PNIPMAM @ IONPs incubated with 1 μ g TRF at pH 7.4 for 30 and 60 min at 45 °C. **b.)** Western blot analysis and quantification of the samples collected after incubation with 10 mg/ml competitor sugars (pH = 7.4) for 60 min at 45 °C (n = 3, error bars denote standard error).

After studying the effect of protein size, PI and glycosylation for the competitor proteins, it was decided to investigate similar properties for the guest proteins. Such information could help us understand better the binding of proteins to the PNIPMAM @ IONPs, which would be advantageous in developing protein cargoes for further applications.

5.5. Change in guest proteins

To investigate the effect of protein properties on their encapsulation, a smaller non-glycosylated green fluorescent protein (GFP, 27 kDa), and a larger glycosylated protein bovine IgG (160 kDa) were selected. 0.5 mg of PNIPMAM @ IONPs were incubated with 1 μ g of guest proteins (GFP, IgG) followed by separation of the NPs from the unbound protein using

centrifugation. The supernatant was collected and analysed to quantify the amounts of unloaded guest protein (**Fig. 5.11**).

Due to smaller size than TRF (80 kDa) and the absence of glycosylation, weaker interactions between GFP and NPs were expected. For the significantly bigger IgG (glycosylated), higher protein encapsulation than TRF was anticipated. However, the results were not as expected (**appendix K, N**), the protein encapsulation reactions gave different results, loading was better for both GFP (~ 90 % of 1000 ng) and IgG (~ 80 %) as compared to TRF (~ 40 %) (**Fig. 5.12**). In addition to encapsulation efficiency, we also investigated the triggered release of these guest proteins as described in the next section.

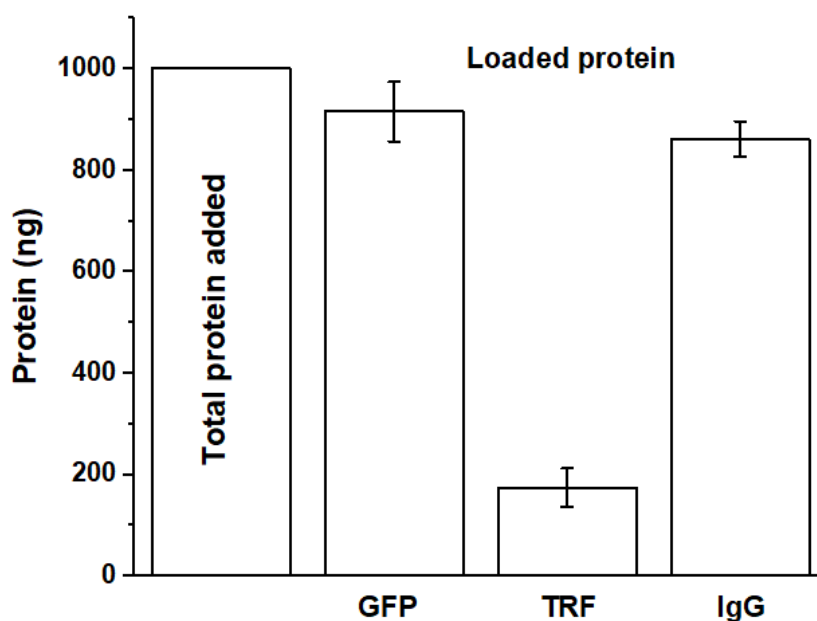
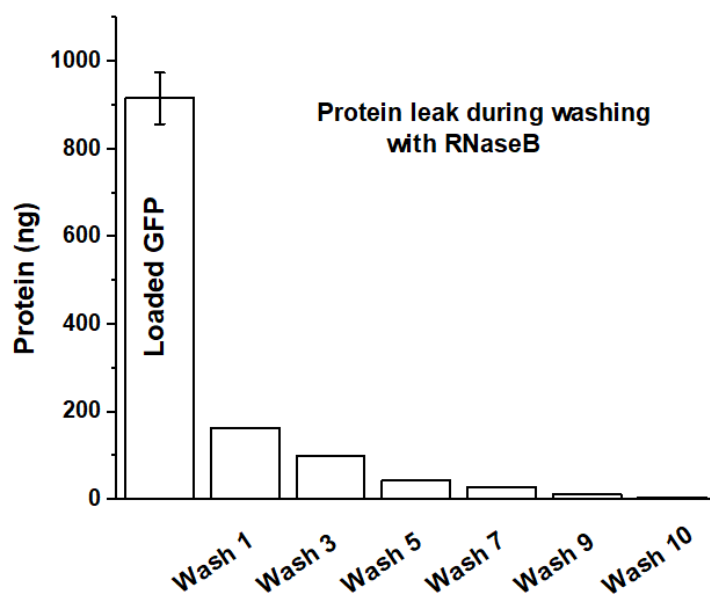


Fig. 5.12. Loading of different competitor proteins (GFP, TRF and IgG) to PNIPMAM @ IONPs: Western blot analysis (**appendix K, M**) and quantification of the supernatant was done to quantify unloaded TRF and IgG. All GFP samples were analysed using a spectrofluorometer (96-well plate reader) and quantified using a GFP standard curve (**appendix O**). (n = 3, error bars denote standard error).

5.5.1. Triggered release of GFP from PNIPMAM @ IONPs

Before checking triggered release of guest proteins from PNIPMAM@IONPs, protein loaded NPs were washed several times with the competitor protein solution (0.07mM in 20mM HEPES + 100mM NaCl, pH = 7.4). These washes were performed to remove any loosely bound guest protein from PNIPMAM@IONPs. NPs were centrifuged, the supernatant collected, and analysed (labelled as wash samples in **Fig. 5.13** (a, b)).

a)



b)

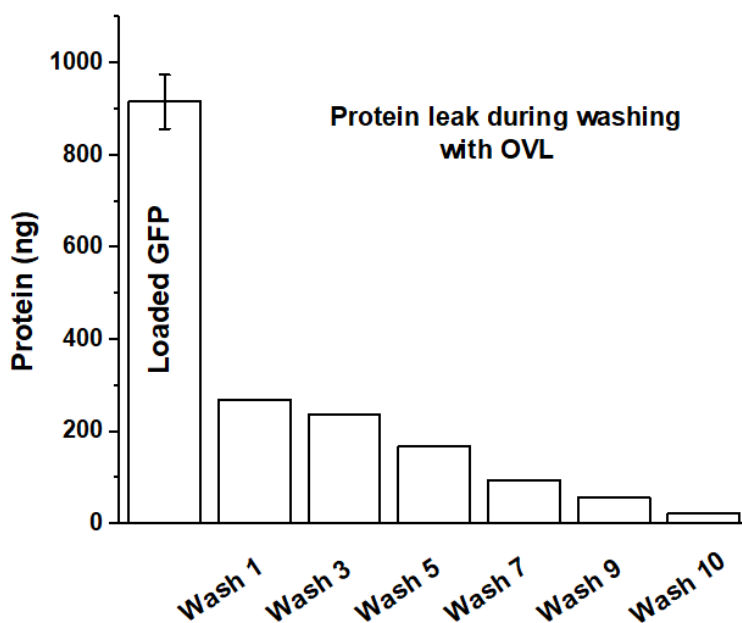


Fig. 5.13. Washing of GFP loaded NPs with different competitor proteins: a) RNaseB and b) OVL. After loading GFP, NPs were washed multiple times with 10 mg/ml competitor proteins at pH 7.4 to remove any non-specifically bound protein. All GFP samples were analysed using a spectrofluorometer (96-well plate reader) and quantified using a GFP standard curve (**appendix O**). (n = 3, error bars denote standard error).

After TRF loading, the majority of the loosely bound protein was removed with a few competitor protein washes (**appendix I**). However, for GFP loaded NPs, significant protein loss was observed during initial RNaseB washes followed by a constant leak towards the end. There was a ~ 3-fold increase in the amount of protein leak when bigger OVL was used as competitor (**Fig. 5.14**). The release triggered by polymer collapse at 45°C for GFP loaded PNIPMAM @ IONPs with RNaseB and OVL did not differ from the control experiment samples collected at 37°C, below the polymer transition temperature. This implied that the small non-glycosylated GFP protein gets encapsulated well to PNIPMAM @ IONPs but then leaches very well too. We hypothesized that this could either be due to the smaller size of GFP or the absence of glycans in its structure.

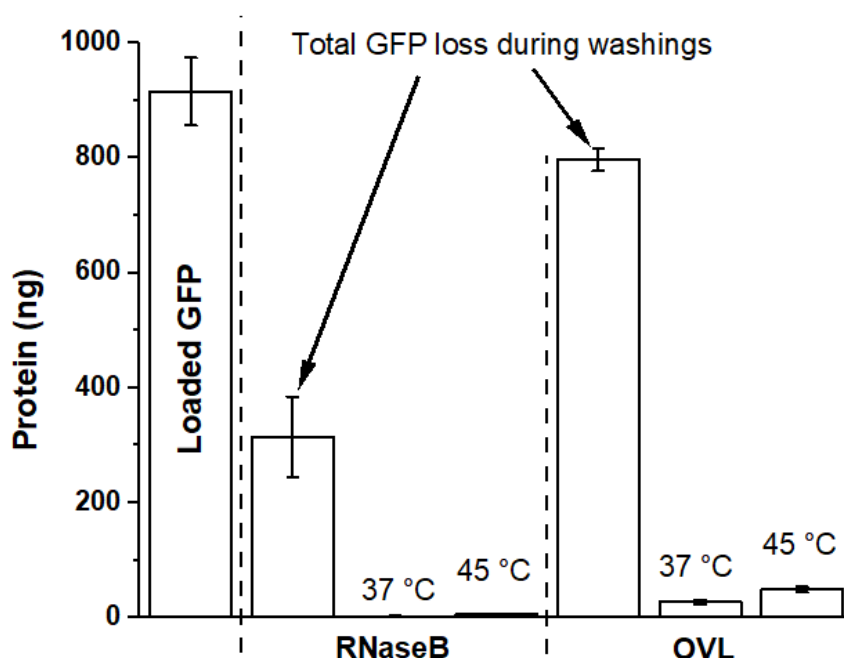


Fig. 5.14. PNIPMAM @ IONPs release GFP in a temperature-dependent manner with different competitor proteins: Quantification following 10 mg/ml competitor protein

treatment (RNaseB and ovalbumin) of 0.5 mg PNIPMAM @ IONPs incubated with 1 μ g GFP at pH 7.4 for 60 min at 37 °C and 45 °C (n=3, error bars denote standard error). Protein release was calculated relative to the amount of GFP associated with the particles after 10 washings. All GFP samples were analysed using a spectrofluorometer (96-well plate reader) and quantified using a GFP standard curve (**appendix O**). (n = 3, error bars denote standard error).

The experimental findings for the triggered release of IgG (160kDa) could clarify this hypothesis and are reported in the next section.

5.5.2. Triggered release of IgG from PNIPMAM @ IONPs

Before checking triggered protein release, IgG loaded PNIPMAM @ IONPs were subjected to competitor protein washings. Three different competitor proteins were used; RNaseB, OVL and TRF. Similar to the TRF encapsulation, most of the unbound IgG was removed during initial competitor protein washes (3-6 washes). For the triggered release with the competitor proteins, a clear protein release was observed above the LCST. A similar size behaviour as TRF release was observed where, higher amount of IgG was released (**Fig. 5.15**) with bigger competitor proteins (OVL and TRF). Irrespective of their size difference, similar amounts of IgG and TRF (~ 150 ng) were released with OVL. It was also indicating the complexity of guest/competitor protein - NPs interactions in the triggered protein release.

In conclusion, protein encapsulation/triggered release from PNIPMAM @ IONP is a combined effect of protein-NP and protein-polymer interactions. Below the LCST, protein encapsulation to NP could be due to H-bonding between protein and polymer functionalities, and protein-metal chelation with the iron oxide core. Whereas, above the LCST, the interactions between NP-protein would be mainly hydrophobic in nature. These interactions are mainly affected by the protein size, PI and glycosylation where, glycosylated proteins bind better to the NP surface than non-glycosylated proteins. TRF release experiments with various saccharides also revealed some affinity of the NP to the glycan (mannose), and hence could be the reason for the preferential binding of the glycoprotein to PNIPMAM @ IONP.

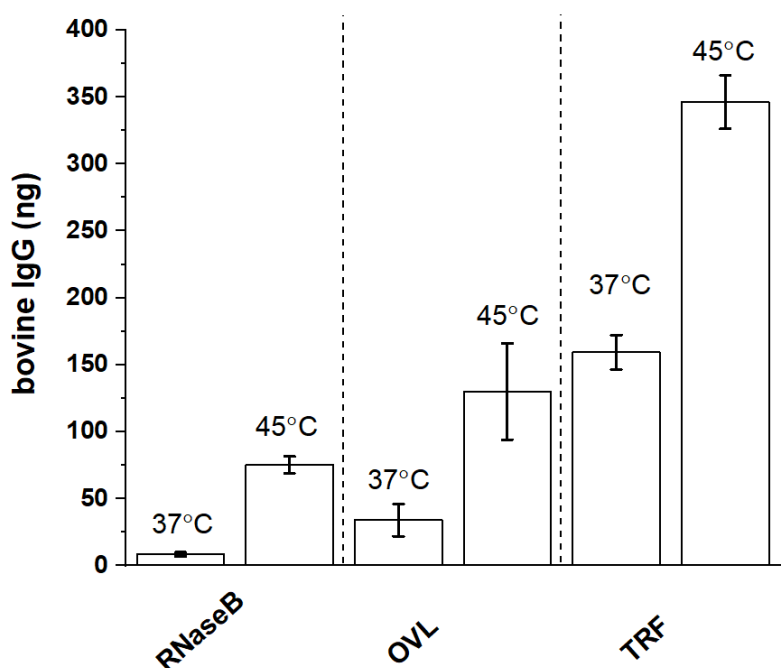


Fig. 5.15. PNIPMAM @ IONPs release IgG in a temperature-dependent manner with different competitor proteins: Western blot analysis (**appendix N**) and quantification following 10 mg/ml competitor protein treatment (RNaseB, OVL, TRF) of 0.5 mg PNIPMAM @ IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 37 °C and 45 °C (n = 3, error bars denote standard error).

In future *in vivo* applications of the coated NPs, growth medium for cell and tissue culture contains serum (10% fetal bovine serum containing media) as a source of growth supplements. Serum is the fluid and solute component of blood, and contains all the electrolytes, hormones, antibodies and other proteins, except those used in blood clotting.¹⁹ During *in vivo* studies, serum can be used as a potential source of competitor proteins for the triggered guest protein release from PNIPMAM @ IONPs. It would be an ideal mimic of the cell surroundings for studying triggered protein release in a physiological system. Therefore, it was decided to investigate serum as a competitor in triggered protein release and the findings are reported in the next section.

5.6. Serum as a competitor

We started our experiments by studying the reactivity of the orthologs of our chosen guest proteins (TRF and IgG) present in various serums (bovine, goat and pig serum) to the antibody used for detecting the used guest protein on western blots. The main aim for these experiments was to select a suitable guest protein/serum system, in which the guest protein orthologs in the serum would not be detected by the antibody used on the western blots. The absence of reactivity of these serum proteins with the guest protein specific antibodies was obligatory for the detection of any guest protein release in the presence of serum. During the screening, TRF antigens in all three serums were detected by the antibodies used for the detection of human TRF (**Fig. 5.16**). Hence, TRF was not an ideal guest protein for assessing serum as a source of competitor protein.

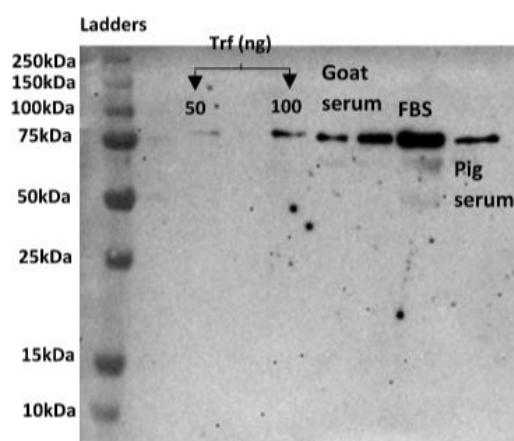


Fig. 5.16. Reactivity of the anti-human TRF antibody against TRF orthologs found in various sera: fetal bovine serum (FBS), goat serum and pig serum were screened with anti-human TRF primary and HRP conjugated goat anti rabbit secondary antibodies for TRF detection. 10 μ L of the 10 % FBS, pig serum and goat serum are loaded with various amounts of pure TRF.

However, the anti-bovine IgG antibody did not recognise the antibodies present in goat serum (**Fig. 5.17**). The anti-bovine IgG was generated in a goat host. It would therefore be expected to be inactive against all the goat self-antigens, including goat IgG. Therefore, for further studies with serum as a competitor, bovine IgG/goat serum was selected as a guest protein/competitor system.

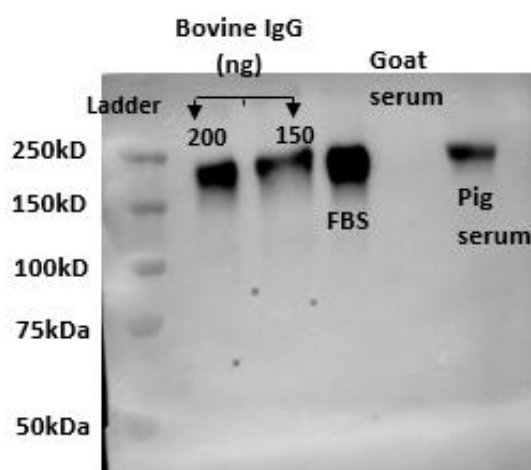
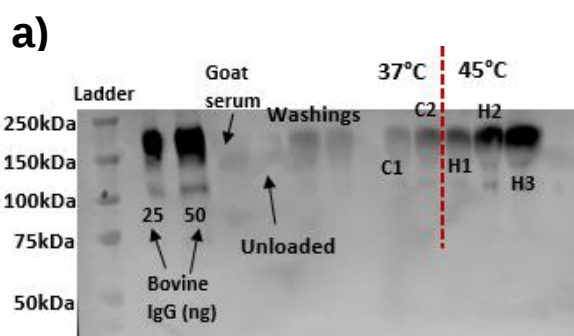


Fig. 5.17. Specificity of the IgG antigens present in various serums (10 %) to the goat anti bovine IgG antibody: fetal bovine serum (FBS), goat serum and pig serum. 10 μ L of the 10 % FBS, pig serum and goat serum are loaded with various amounts of pure bovine IgG.

After IgG loading, control and heating experiments were done on the protein loaded NPs with goat serum as a competitor (10 % in 20mM HEPES, pH 7.4). Serum contains a mixture of complex proteins which vary in their size, IP and glycosylation. It was expected that the bigger proteins in the serum would dominate the triggered release and most of the encapsulated IgG would be released above the LCST. However, the protein release was similar to the OVL competitor. We were releasing ~ 100 ng of IgG at 45 $^{\circ}$ C with a significant leakage of ~ 30 ng at 37 $^{\circ}$ C (**Fig. 5.18 (a, b)**). Since serum is a complex blend of proteins and electrolytes, it isn't credible to comment anything on its interactions with the PNIPMAM@IONPs. However, these results were suggesting that during *in vivo* studies, the serum can be used as a potential source of competitor proteins for the triggered protein release.



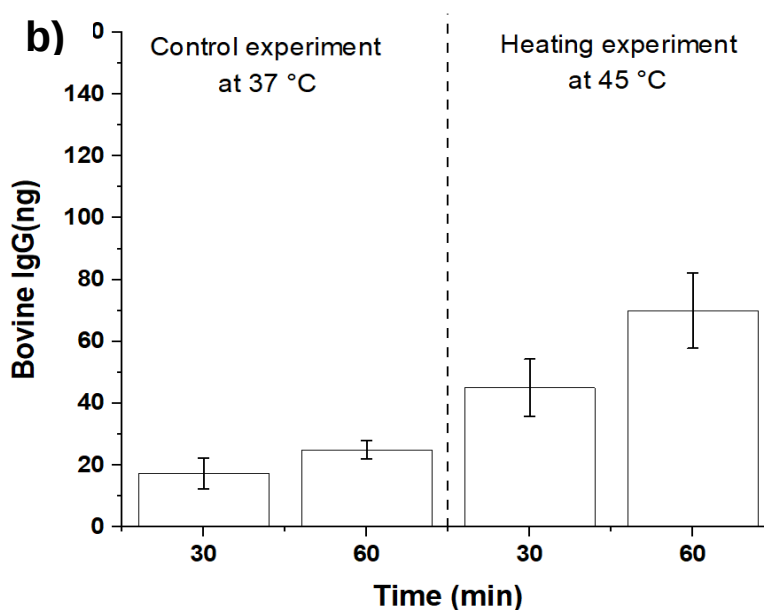
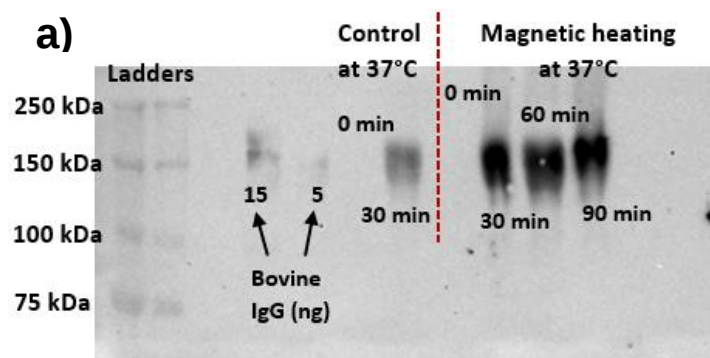


Fig. 5.18. PNIPMAM@IONPs release IgG in a temperature-dependent manner: a.) Western blot analysis following 10 % goat serum treatment of 0.5 mg PNIPMAM @ IONPs incubated with 1 μ g IgG at pH 7.4 for 30 min (C_1 and H_1) and 60 min (C_2 and H_2) at 37 °C and 45 °C, respectively. **b)** Quantification of IgG release from PNIPMAM @ IONPs at 37 °C and 45 °C, respectively ($n = 3$, error bars denote standard error). Protein release was calculated relative to the amount of IgG associated with the particles after 10 washings.

After checking serum for the triggered IgG release in a temperature-dependent manner, it was decided to investigate magnetically triggered protein release. We wanted to assess whether magnetic heating would have a similar effect as thermal heating or if it would improve the IgG release. To investigate this, we used a temperature-controlled room (37 °C) to maintain a constant body temperature environment. Some control measures were taken to assess magnetic heating of PNIPMAM @ IONPs. An infrared thermocouple (gallium arsenide) was used to monitor the sample temperature in real time. The thermocouple did not contain any trace magnetic material so would not respond to alternating current induced magnetic field (AMF). It was immersed in the sample volume environment through an adapted plastic inlet tube. Hence it would not contribute to any magnetic heating observed. Additionally, to avoid any background heating, water was pumped through the coils around the magnet to dissipate the extra heat. Before experimentation, a control sample without NPs was exposed to the magnetic field for 30 min (1 min ON 30 sec OFF) to check for any background heating.

With the magnetic heating experiments, we could show that the encapsulated IgG was released from PNIPMAM @ IONPs after 30 min of AMF. Interestingly, triggered IgG release with magnetic heating was quicker as compared to conventional heating. Protein release for 30 min with magnetic heating was higher (~ 90 ng, **Fig. 5.19 (a, b)**) as compared to the conventional heating (~ 30 ng, **Fig. 5.18 (a, b)**). However, the total protein release levels (after 60 min) were constant (~ 100 ng) for both magnetic and conventional heating. Importantly, the magnetically triggered release was observed while the bulk solution temperature was below the LCST of PNIPMAM @ IONPs (38.3 °C). The local heating on the NP shell would be much higher as compared to the surrounding environment. It means that the heating of NP core could result in protein release when the bulk temperature is well below the LCST. Perhaps the local heating of the core resulted in better heating of the protein-loaded polymer shell, which could be the reason for faster protein release as compared to conventional heating. But these are just speculations as it would be difficult to assess the core temperature during magnetic heating, which could be an interesting future project.



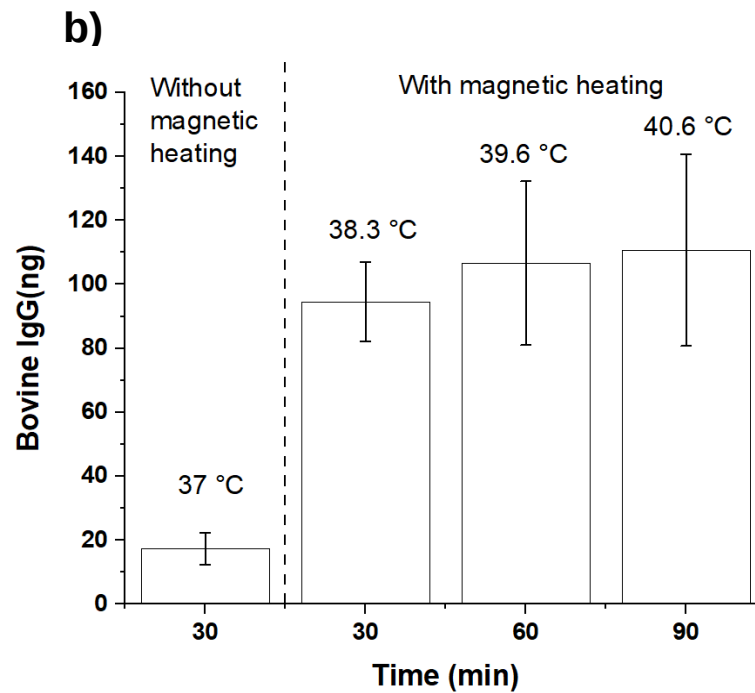
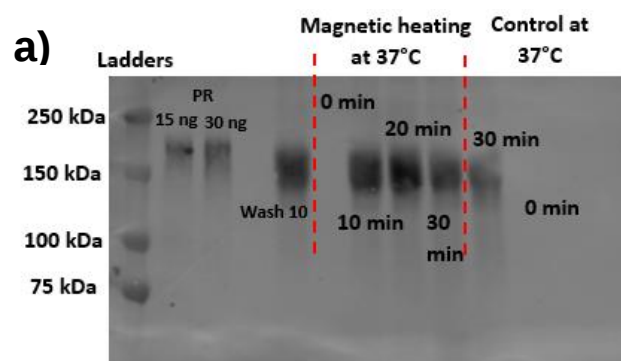


Fig. 5.19. Magnetic heating triggered IgG release from PNIPMAM @ IONPs at 37 °C: a.) Western blot analysis following 10 % goat serum treatment of 0.5 mg PNIPMAM @ IONPs incubated with 1 µg IgG at pH 7.4 for 0, 30, 60 and 90 min at 37 °C with and without magnetic heating (1 min on / 30 sec off). **b)** Quantification of IgG release from PNIPMAM @ IONPs with magnetic heating (n = 3, error bars denote standard error). Temperatures during magnetic heating are provided above each timepoint. Protein release was calculated relative to the amount of IgG associated with the particles after 10 washings.



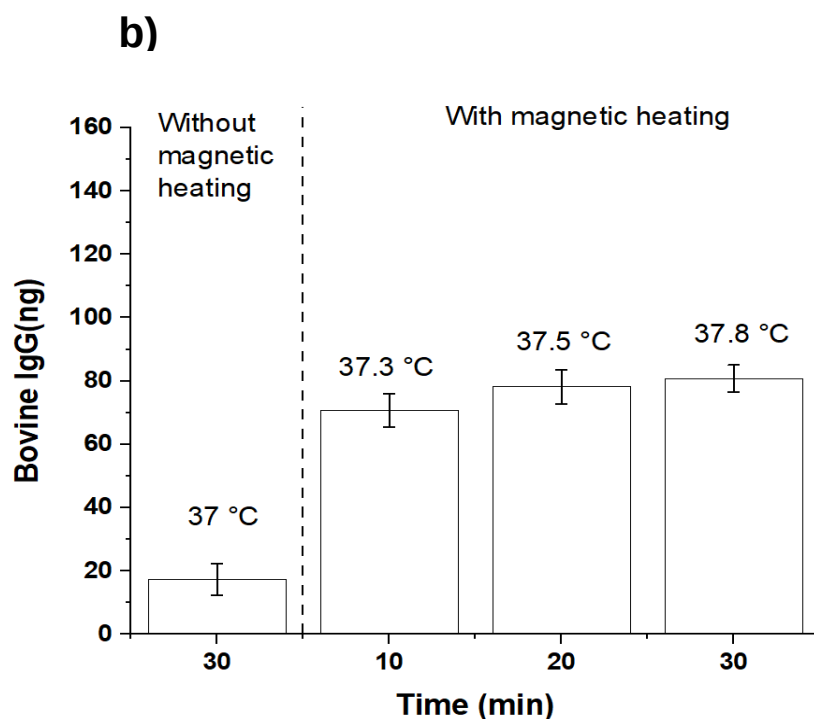


Fig. 5.20. Pulsed magnetic heating triggered IgG release from PNIPMAM @ IONPs at 37°C:
a.) Western blot analysis following 10 % goat serum treatment of 0.5 mg PNIPMAM@IONPs incubated with 1 μ g IgG at pH 7.4 for 0, 10, 20 and 30 min at 37 °C with and without magnetic heating (10 sec on / 30 sec off). **b)** Quantification of IgG release from PNIPMAM@IONPs with magnetic heating ($n = 3$, error bars denote standard error). Temperatures during magnetic heating are provided above each time point. Protein release was calculated relative to the amount of IgG associated with the particles after 10 washings.

After observing IgG release below the LCST of the PNIPMAM @ IONPs following magnetic heating, it was decided to investigate it further with milder AMF conditions (**Fig. 5.20** (a, b)). Using pulsed AMF (10 sec ON 30 sec OFF) for 30 min, there was only a very small increase in the bulk solution temperature (37.8 °C). However, the amount of protein release was still significant, and reduced only to ~ 80 ng as compared to the harsher AMF (~ 90 ng, **Fig. 5.21**). This supports our previous observations suggesting that the magnetic triggered protein release from PNIPMAM @ IONPs is mostly governed by the local heating of the NPs and is independent on the bulk sample temperature exceeding the LCST.⁷

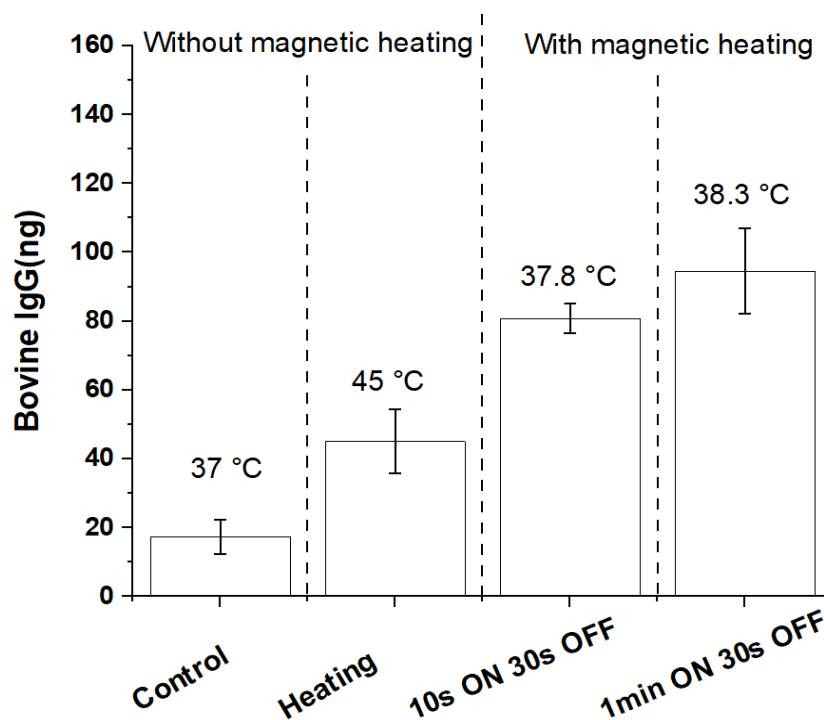


Fig. 5.21. PNIPMAM @ IONPs release IgG in a temperature-dependent manner with and without magnetic heating with goat serum (10 %) as competitor: Quantification of IgG release from PNIPMAM @ IONPs with and without magnetic heating (n = 3, error bars denote standard error) for 30 min. Temperatures during magnetic heating are provided above each time point.

5.7. Conclusions

Thermo-responsive PNIPMAM @ IONPs were studied as a potential system for the protein encapsulation/release in a temperature-dependent manner. Effective protein encapsulation was achieved by exploring the phase transition properties of the core-shell NPs. H-bonding (below the LCST), and hydrophobic interactions (above the LCST) could be major contributors for the protein encapsulation to the core-shell NP. There was some unknown effect of PI too as both protein and NP were charged. Bigger proteins (OVL) were more efficient competitors than the smaller ones (RNaseB). Additionally, glycosylated proteins (RNaseB) outperformed non-glycosylated competitors (RNaseA) in the triggered protein release suggested possible NP-glycan interactions. Release experiments with saccharides showed the specificity of the NP-glycan interactions in the glycoproteins.

Magnetic heating of the PNIPMAM @ IONPs was also explored as a potential thermal trigger to release the encapsulated protein from the core-shell NP. Serum was used as a competitor protein source. The encapsulated protein release with a pulsed AMF application (1 min ON 30 sec OFF) was quicker as compared to the release with the conventional heating. Moreover, with AMF, heating of the bulk sample temperature above the LCST was not required for the triggered protein release. This possibly suggests that the temperature-dependent protein release from PNIPMAM @ IONPs is largely influenced by the NP surface environment during magnetic heating. Higher temperature at the NP surface could be the reason for PNIPMAM collapse and triggered protein release. Following a mild pulsed AMF application (10 sec ON 30 sec OFF) for 30 min, protein release was obtained at even lower temperature (37.8 °C) than the first pulsed AMF regime (38.3 °C, 1 min ON 30 sec OFF) and the protein release was still rapid as compared to the conventional heating. These results suggested high magnetic heating efficiency of our core-shell NP system and hence, PNIPMAM @ IONPs are a potential candidate for protein delivery and release above the physiological temperature.

5.8. References

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Chapter 6: Overall conclusions and future work

In this work, we have successfully developed PNIPMAM surface-functionalised IONPs that were both magnetically-responsive and temperature-sensitive. The LCST of the core-shell NPs was tuned by using different M_w PNIPMAM, where the phase transition temperature increased with increase in polymer's chain length. After getting core-shell NPs with a tuned LCST, their diffusion behaviour was studied through agar hydrogel. The diffusion coefficient for different M_w PNIPMAM @ 15.4 ± 2.1 nm IONPs was similar and hence suggested that the diffusion of core-shell NP could be an interplay between size and weak interactions with the gel fibres. These results also showcased the possibility of the NP-saccharide interactions in the biological media. Optimization of the core-shell NPs for the encapsulation/triggered release of the model test protein apotransferrin (TRF) revealed the potential of 40 kDa PNIPMAM @ 15.4 ± 2.1 nm IONPs for the protein delivery applications. H-bonding (below the LCST), and hydrophobic interactions (above the LCST) could be major contributors for the protein encapsulation to the core-shell NP.

This NP system was tested with different competitor proteins, where the triggered TRF release was higher for bigger glycosylated competitors than the non-glycosylated proteins. This could be due to the preferential binding of the glycoprotein to the NPs which further showcased the possibility of NP-glycan interactions. These interactions were further tested by screening various saccharides as competitors for the triggered TRF release where, a small amount of protein release was observed only with D-mannose. Since most of the glycoproteins used in this study are mannose rich, this specificity could enhance their attachment to the core-shell NPs than the non-glycosylated proteins.

Serum was also explored as a competitor protein source for the magneto-thermal protein release from PNIPMAM @ IONP. The encapsulated protein release with a pulsed AMF application was achieved below the LCST of core-shell structure. This suggested that the temperature-dependent protein release from PNIPMAM @ IONPs is largely influenced by the NP surface environment during magnetic heating. Therefore PNIPMAM @ IONPs are a

potential candidate for the protein delivery/magneto-thermal release above the physiological temperature.

Irrespective of a higher LCST ($\sim 45\text{ }^{\circ}\text{C}$) of 40 kDa PNIPMAM @ $15.4 \pm 2.1\text{ nm}$ IONPs, there was still some protein leak at physiological temperature ($37\text{ }^{\circ}\text{C}$), which is not ideal for their *in vivo* applications. Therefore, more insight on the NP-protein interactions is required to improve our protein carrying cargo. Micro differential scanning calorimetry (MicroDSC) and isothermal titration calorimetry (ITC) are the common techniques used for the detection of the nature of NP-ligand interactions. Hence the potential future directions for this work could involve DSC and ITC studies on NP-protein interactions. This could help us decode the nature of NP-protein interactions and hence design leak proof protein carrying cargo. As we previously reported the use of PNIPAM @ IONPs in the triggered release of Wnt3a to the mesenchymal stem cells (MSCs), PNIPMAM @ IONPs could also be tested for similar applications. Additionally, specific targeting could be achieved by functionalizing PNIPMAM @ IONPs with target specific antibodies that recognises specific cell marker of the MSCs. However, it would be important to assess the *in vivo* magneto-thermal protein release to assess how effective magnetic nanoparticle heating is through tissue. Whether magnetically-triggered protein release will be effective in a more complex biological environment remains to be seen, but certainly should be addressed.

Chapter 7: Experimental

7.1. Materials

All chemicals and solvents were used as received unless mentioned otherwise.

Different chain length PNIPMAM were synthesized using atom transfer radical polymerization (ATRP) and reversible addition-fragmentation chain transfer (RAFT) polymerization. For PNIPMAM synthesis using ATRP, *N*-isopropylmethacrylamide (NIPMAM) was purchased from Sigma-Aldrich; tris[2-(dimethylamino)ethyl]amine (Me₆TREN) from TCI; 2-methyl-2-bromopropionic (MPA) acid and Cu(I)Br were purchased from Alfa Aesar. To synthesize PNIPMAM using post functionalization ATRP approach, *N,N,N',N'',N''*-pentamethyldiethylenetriamine (PMDETA) and ethyl 2-bromoisobutyrate (EBIB) were purchased from TCI and dry anisole from Acros Organics. *N*-succinimidyl methacrylate (MASI) monomer was synthesized in house.

For RAFT polymerization, chain transfer agent (CTA) 2-(dodecylthiocarbonothioylthio)-2-methylpropionic acid was purchased from Fluorochem and the radical initiator azobisisobutyronitrile (AIBN) was purchased from Sigma-Aldrich. For nitrodopamine synthesis (NDA), dopamine hydrochloride and sodium nitrite were purchased from Alfa Aesar. For the functionalization of PNIPMAM with NDA, coupling agent (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and *N,N*-diisopropylethylamine (DIPEA) were purchased from Sigma-Aldrich. For the IONPs synthesis, iron(III)acetylacetonate (Fe(acac)₃), oleyamine (OAm), 1,2-tertadecanediol, decanoic acid and benzyl ether were purchased from Sigma-Aldrich. Oleic acid (OA) and 4-biphenyl carboxylic acid were purchased from Alfa Aesar.

Water used for lower critical solution temperature (LCST) studies was purified using a Millipore Milli-Q system with a QPAK 2 column. All syringe injections for water and air sensitive reactions were made using a syringe purged with nitrogen gas three times immediately prior to use.

7.2. General characterizations

UV-Vis spectroscopy was conducted across a wavelength range of 300-800 nm, recorded on a Shimadzu UV-1800 UV-Vis spectrophotometer. Quartz cuvettes were used with a 1 cm pathlength and samples were dissolved in 1 mL sodium citrate (1 mM, pH 5.5) made up in dH₂O for analysis.

¹H-NMR (400 MHz) spectra were obtained in CDCl₃, D₂O or DMSO-d₆ on a JEOL ECS-400 spectrometer using 20 mg/mL and 50 mg/mL samples for ¹H and ¹³C NMR, respectively. A 400 MHz field strength was used with 8 scans across a scan range of -2 to 12 ppm. For ¹H NMR of polymers, a 400 MHz field strength was used with 128 scans across a scan range of -2 to 12 ppm.

Molecular weight (M_n) characterisation was conducted using Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (Ultraflex, Bruker) in positive, linear ion mode. 1 mg/mL samples were spotted (2 μ L) at dilutions of 1/10, 1/100 and 1/1000, made up in a 20 mg/mL matrix solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) in THF.

Polymer LCST was measured by nano differential scattering fluorimetry (NanoDSF) method conducted on Prometheus NT.48 with a ramp rate of 0.4 °C/min. Polymer samples were prepared in dH₂O and loaded in the NanoDSF capillaries (standard, NanoTemper).

For the size determination of IONPs, TEM images were obtained using a JEOL 2011 transmission electron microscope operated at 200 kV accelerating voltage. CCD images were extracted using Gatan Digital Micrograph software. Prior to analysis, 1 mg/mL ([Fe]) samples were prepared in toluene by sonication for 15 min. The suspension was further diluted (1 in 10) with toluene and one drop of the dispersion was deposited onto 3 mm holey carbon coated copper grids, which were allowed to dry in air prior to analysis.

XRD patterns were obtained from a Bruker D8 powder diffractometer equipped with a Cu source (Cu K α wavelength = 1.5406 Å) at a scan rate of 0.121 min⁻¹ (100 mg dry and finely powdered sample).

Hydrodynamic diameter and zeta potential measurements were recorded using a Zetasizer and analysed using the DTS v. 5.1 supplied by Malvern. 1 mg/mL ([Fe]) samples were prepared by sonication in dH₂O for 15 min before placing sample (1 mL) into a disposable DLS cuvette for size distribution and a U-bend cell for zeta potential. Measurements were made at 25 °C and carried out in triplicate at 10 runs per measurement.

In the next section, experimental procedures for various PNIPMAM and IONPs synthesis followed by protein studies are briefly described along with some of the above-mentioned techniques in details.

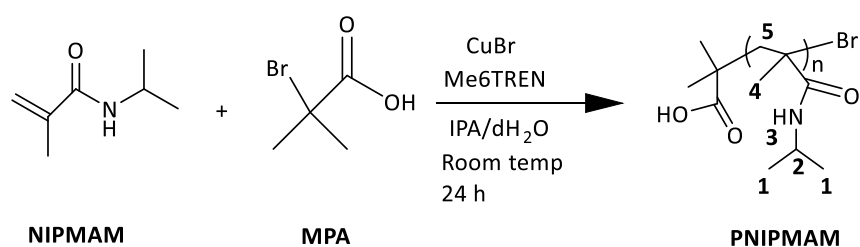
7.3. Experimental procedures from Chapter 2

7.3.1. Synthesis of various chain length PNIPMAM

Different molecular weight PNIPMAM (3 kDa – 89 kDa) were synthesized using ATRP and RAFT polymerizations. For ATRP, two different approaches were used; direct conversion from monomer¹ and post-functionalization approach.² Experimental methodologies for both approaches followed by RAFT synthesis procedures are reported in the next section.

7.3.1.1. Synthesis of PNIPMAM using ATRP

7.3.1.1.1. Direct conversion of NIPMAM to PNIPMAM



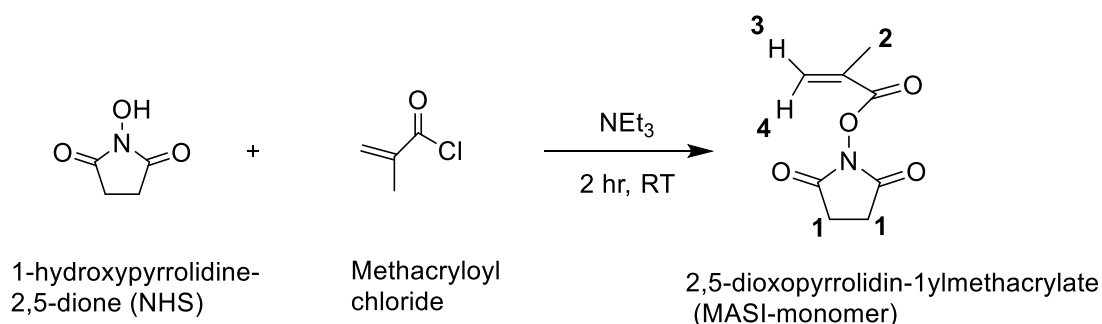
Scheme 7.1. Reaction scheme for PNIPMAM synthesis using ATRP.

PNIPMAM was synthesised by an adapted literature procedure.¹ For a 5 g scale reaction (**scheme 7.1**), NIPMAM (5 g, 39.3 mmol), Cu(I)Br (112.75 mg, 0.786 mmol) and initiator MPA (131.26 mg, 0.786 mmol) were weighed in a round bottomed flask (RBF, 50 mL). The RBF was sealed with a septum and fitted with a nitrogen balloon to provide inert conditions during the addition of other reagents. Nitrogen-purged isopropanol (10 ml) was added to the RBF and the mixture was further purged (N₂) for 15 min. The ligand Me₆TREN (1 ml) was purged with nitrogen for 20 min and then injected (420 µL, 1.572 mmol) into monomer solution to start the polymerization. After the ligand addition, the reaction mixture was stirred for 6 h at room temperature in a water bath. After 6 h, the RBF was opened to air and the IPA was removed on a rotatory evaporator at 60 °C. The crude solid was then dissolved in 10 ml THF and the polymer was separated by precipitation in n-pentane (250 ml). The polymer was collected by centrifugation and purified by redissolving and precipitating in THF:n-pentane (1:10) three times before drying on the rotatory evaporator at 40 °C. Yield: 2.4 g (48 %). ¹H NMR: (D₂O, 400 MHz), δ (ppm) = 6.9-7.3 (brm 1H, **3**), 3.6-3.9 (brm, 1H, **2**), 1.4-2.0 (brm, 2H, **5**), 0.5-1.1 (brm, 6H, **1**), 0.5-1.1 (brm, 3H, **4**).

7.3.1.1.2. Synthesis of PNIPMAM using post-functionalization approach

7.3.1.1.2.1. Synthesis of the *N*-succinimidyl methacrylate (MASI) monomer

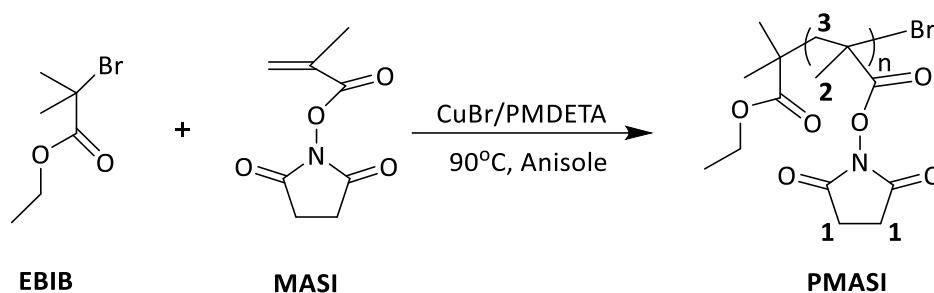
MASI-monomer was prepared by a literature method (**scheme 7.2**).³



Scheme 7.2. Reaction scheme for MASI monomer synthesis

For a 5g scale reaction, *N*-hydroxysuccinimide (5.0 g, 43.4 mmol), triethylamine (7.27 mL, 52.1 mmol), and dichloromethane (DCM) (50 mL) were added to a 250 mL round-bottomed flask (RBF) equipped with a stirring bar. The flask was sealed with a septum, purged with nitrogen and then placed in an ice-bath at 0 °C. Methacryloyl chloride (4.67 mL, 47.8 mmol) was added drop-wise to the system under inert condition with vigorous stirring. The reaction was stirred for 2 h under nitrogen and then warmed to room temperature. DCM (20 mL) was added to the reaction mixture and the reaction mixture was filtered to remove triethylammonium chloride formed as a reaction by-product. The organic filtrate was washed with 200 mL aqueous solution of 1 wt% NaHCO₃, washed twice with distilled water and then dried using MgSO₄. The organic layer was concentrated under reduced pressure and 30 mL 1:1 ethyl acetate/hexane mixture was then added. The solution was allowed to crystallize at 4 °C overnight. The product, MASI monomer, was isolated via filtration, washed with hexane, dried under vacuum and stored in freezer. Yield: 3.14 g (63 %). ¹H-NMR: (CDCl₃, ppm) δ = 6.4 (s, 1H, **4**), 5.9 (s, 1H, **3**), 2.8 (s, 4H, **1**), 2.0 (s, 3H, **2**).

7.3.1.1.2.2. Synthesis of poly(*N*-succinimidyl methacrylate) (PMASI) using ATRP



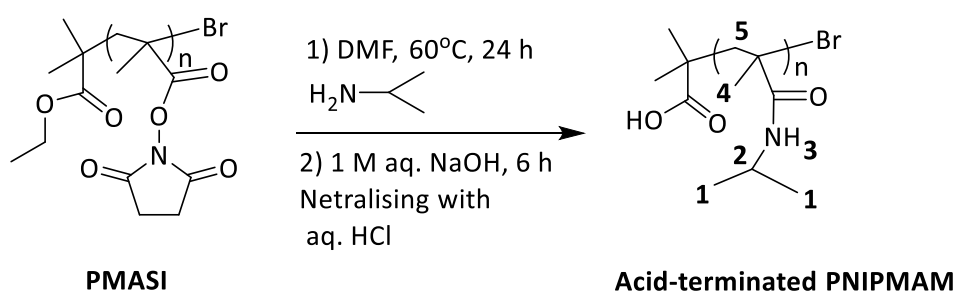
Scheme 7.3. Copper-catalysed homo-polymerization of MASI monomer in anisole

Synthesis was adapted from a previous study (**scheme 7.3**).² For a typical polymerization, MASI (2 g, 10.92 mmol) and CuBr (35.6 mg, 0.25 mmol) were added to a 25 mL RBF equipped with magnetic stirrer bar and vacuum dried for 1 h to remove any moisture present in the system. After drying, RBF was sealed with a septum and purged with nitrogen for 15 min. Simultaneously, dry anisole was also purged with nitrogen for 15 min and then added (14 mL) to the round-bottomed flask with the help of a nitrogen purged syringe. The mixture was

stirred for 15 min, and purged again with nitrogen for 15 min. After dissolving, the solution was purged again with nitrogen for 15 min. In parallel, PMDETA (52 μ L, 0.25 mmol) and EBIB (40 μ L, 0.25 mmol) were added to a separate vial sealed with a septum and purged with nitrogen gas. 1 ml of dry anisole was added to both the vials under inert conditions (using a nitrogen purged syringe), mildly agitated to mix and bubbled with nitrogen for further 10 min. The PMEDTA solution was then added to the round-bottomed flask under inert conditions (using a nitrogen purged syringe) and left to stir at room temperature for 20. After the addition of the EBIB solution to the RBF under inert conditions (using a nitrogen purged syringe), the reaction mixture was placed in a 90 °C-oil bath for 1 h. The crude product was concentrated under reduced pressure on a rotary evaporator at 40°C. The crude mixture was then dissolved in a minimum amount of DMSO (~ 3 mL) and precipitated by pouring in ice-cold acetone (150 mL). The mixture was eventually added to four 50 mL Falcon tubes for centrifugation (4400 rpm, 20 min). The supernatant was removed and the polymer pellets were washed twice with acetone to remove excess DMSO before drying on a rotary evaporator at 40 °C. Yield: 1.04 g (51.8 %). ^1H NMR: (DMSO- d_6 , 400 MHz), δ (ppm) = 2.6-2.9 (brm, 4H, **1**), 1.1 – 1.6 (brm, 2H, **3**), 1.1-1.6 (brm, 3H, **2**).

7.3.1.1.2.3. Synthesis of acid-terminated PNIPMAM from PMASI

Synthesis was adapted from a previous study² (scheme 7.4).

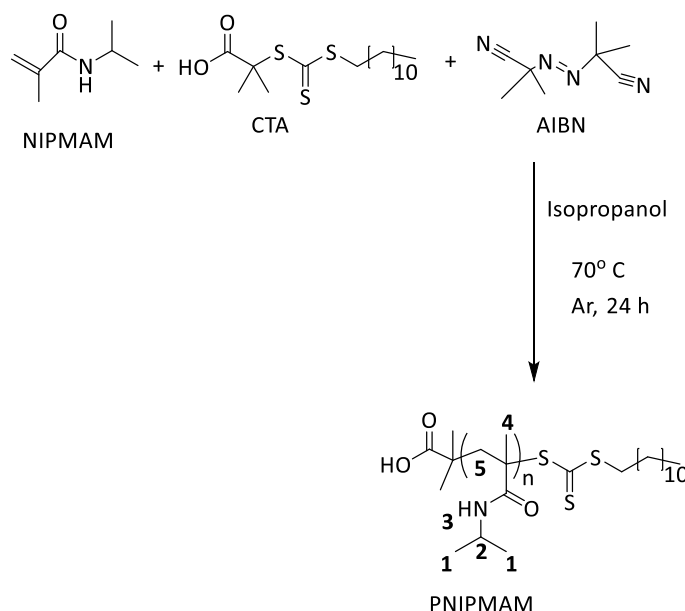


Scheme 7.4. Synthesis of acid-terminated PNIPMAM from PMASI.

For a 674 mg scale reaction, PMASI (674 mg, 0.09 mmol) was added to a 25 mL round-bottomed flask and dissolved in minimal DMF (8 ml). After that, the flask was sealed and purged with nitrogen gas for 15 min. Simultaneously the IPA-containing vial was purged with

a nitrogen containing balloon for 15 min also. Excess IPA (1 ml) was then added under inert conditions (using a nitrogen purged syringe) to the round-bottomed flask and left stirring for 24 h at 60 °C on an oil bath. The polymer, PNIPMAM, was precipitated by pouring the reaction volume into diethyl ether (40 mL). The mixture was then added to a 50 mL Falcon tube for centrifugation (4400 rpm, 20 min). Then the supernatant was removed and washed twice with diethyl ether to remove excess IPA before drying on a rotary evaporator at 40 °C. The product was then added to a 25 mL round-bottomed flask equipped with a stirrer bar and dissolved in 1 M aqueous NaOH (10 mL). This solution was left stirring at room temperature for 6 h and then neutralised with hydrochloric acid (HCl). The excess solvent was then evaporated out on a rotary evaporator to obtain acid terminated PNIPMAM. The polymer was then dissolved in a minimal amount of dH₂O (10 mL) and dried on a rotary evaporator at 40 °C. Yield: 532 mg (79 %). ¹H NMR: (D₂O, 400 MHz), ¹H NMR: (D₂O, ppm), δ (ppm) = 6.9-7.3 (brm 1H, **3**), 3.6-3.9 (brm, 1H, **2**), 1.4-2.0 (brm, 2H, **5**), 0.5-1.1 (brm, 6H, **1**), 0.5-1.1 (brm, 3H, **4**).

7.3.1.2. Synthesis of PNIPMAM using RAFT

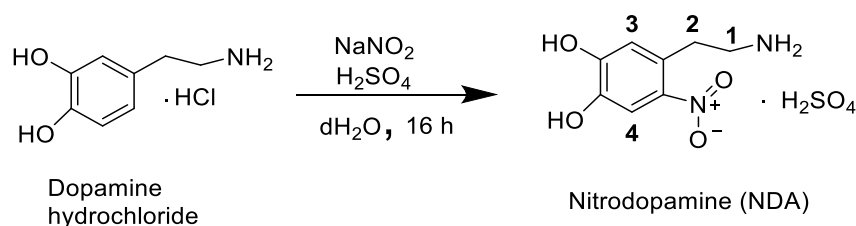


Scheme 7.5. RAFT polymerization of NIPMAM to PNIPMAM

For a 1 g scale reaction (**scheme 7.5**), a mixture of NIPMAM (1 g, 7.86 mmol) and IPA (1 mL) was heated to 70 °C until complete dissolution and cooled to room temperature. CTA (28.7

mg, 0.0786 mmol) in IPA (1 ml) was added to the NIPMAM solution and the reaction mixture was purged with Ar for 1 h. In a separate sample vial, a stock solution of AIBN in IPA (30.4 mM) was purged for 20 min under Ar. 200 μ L (1 mg, 0.006 mmol) of this purged AIBN solution was added to the RBF at 70 °C with vigorous stirring to start the polymerization. After 24 h, the reaction was quenched by cooling to room temperature. IPA was removed on a rotary evaporator and the solid was dissolved in THF (5 ml). PNIPMAM was then selectively precipitated by adding n-pentane (40 ml) and the precipitates were collected by centrifugation (4500 rpm, 10 min). The precipitates were dissolved in THF and combined before evaporating the solvent on a rotary evaporator to get a solid product. Yield: 0.7 g (70 %). ^1H NMR: (D_2O , 400MHz), ^1H NMR: (D_2O , 400 MHz), δ (ppm) = 6.9-7.3 (brm 1H, **3**), 3.6-3.9 (brm, 1H, **2**), 1.4-2.0 (brm, 2H, **5**), 0.5-1.1 (brm, 6H, **1**), 0.5-1.1 (brm, 3H, **4**).

7.3.2. 6-Nitrodopamine hydrogen sulphate (NDA) synthesis

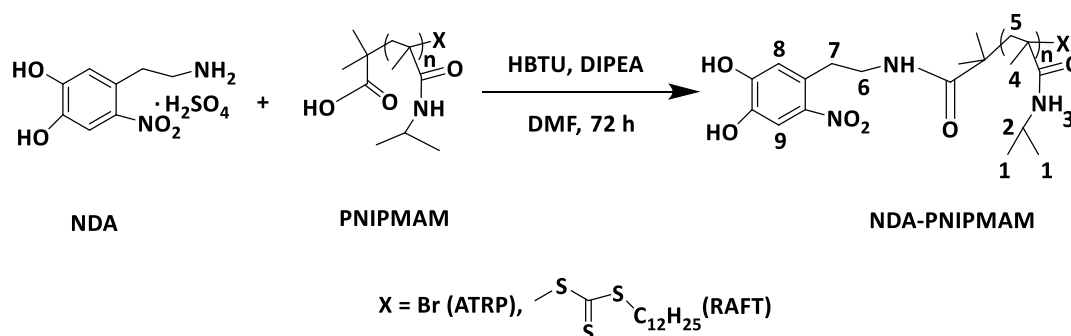


Scheme 7.6. Synthesis route for 6-Nitrodopamine synthesis

A modification of the general procedures reported for nitration of catecholic derivatives⁴ was utilised (**scheme 7.6**). In brief, for a 2 g scale reaction, a solution of concentrated sulphuric acid (960 μ L, 17 mmol) in dH_2O (10 mL) was cautiously added (drop-wise) to a solution of dopamine hydrochloride (2 g, 10 mmol) and sodium nitrite (1.52 g, 22 mmol) in dH_2O (25 mL) cooled in an ice bath. A yellow precipitate formed, and the reaction vessel was left stirring overnight at room temperature. The yellow filtrate was collected by filtration through a Büchner funnel and was washed with water and methanol to afford 2-nitrodopamine hydrogen sulphate. The purification was done by recrystallization in hot (70 °C) dH_2O (50 ml) to yield small yellow crystals which were dried on a rotary evaporator at 40 °C. Yield: 1.12 g

(56 %). ^1H NMR: (D_2O , 400 MHz) δ (ppm) = 7.48 (s, 1H, **4**), 6.87 (s, 1H, **3**), 3.16 (t, J 7.3 Hz, 2H, **1**), 3.05 (t, J 7.3 Hz, 2H, **2**).

7.3.3. NDA functionalized PNIPMAM (NDA-PNIPMAM) synthesis



Scheme 7.7. Synthesis of NDA functionalized PNIPMAM

Acid-terminated PNIPMAM was end-functionalised with NDA through an amide coupling reaction (**scheme 7.7**). Synthesis was adapted from a previous study⁵. PNIPMAM (2g, 0.67 mmol, 3kDa), HBTU (304.91 mg, 0.804 mmol) and DIPEA (116.7 μL , 0.67 mmol) were added to a 50 ml RBF equipped with a magnetic stirrer bar. The mixture was dissolved in dry DMF (19 mL) before sealing the flask with a septum and purging with nitrogen gas for 15 min. The reaction mixture was stirred for 1 h under nitrogen. Subsequently, NDA (398.34 mg, 2.01 mmol) in DMF (1 mL) and DIPEA (233.4 μL , 1.34 mmol) were added under inert conditions, and the resulting solution was stirred at room temperature for 3 days. The solution was acidified with a few drops of 2 M HCl. The polymer was precipitated by dropping the solution in cold diethyl ether (100 ml) and collected via centrifugation. Further purification of the polymer was carried out by dissolving the precipitates in dH_2O (40 ml) and isolated by heating the solution to 60 $^{\circ}\text{C}$ (ATRP PNIPMAM). The precipitates were collected by centrifugation and the washing was done similarly at least twice. After washing twice, the precipitates were dissolved in minimum dH_2O (10 ml) and dried on a rotary evaporator at 40 $^{\circ}\text{C}$. For RAFT polymers, polymer was purified by dissolving in minimum dH_2O (~ 10 ml) followed by centrifugation to remove unreacted NDA. Polymer was further purified using dialysis (2 days), and dried on a rotary evaporator at 40 $^{\circ}\text{C}$. Yield: 1.34 g (67%). ^1H NMR: (D_2O , 400 MHz), δ

(ppm) = 7.5-7.6 (brm, 1H, **9**), 6.9-7.3 (brm, 1H, **3**), 6.6-6.7 (brm, 1H, **8**), 3.6-3.9 (brm, 1H, **2**), 13.48 (brm, 2H, **6**), 3.05 (brm, 2H, **7**), 1.4-2.0 (brm, 2H, **5**), 0.5-1.1 (brm, 6H, **1**), 0.5-1.1 (brm, 3H, **4**).

7.4. Characterization: Molecular weight and phase transition of the PNIPMAM

7.4.1. Molecular weight determination using MALDI-MS

Molecular weight (M_n) characterisation was conducted using Matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (Ultraflex, Bruker) in positive, linear ion mode. 1 mg/mL samples were spotted (2 μ L) at dilutions of 1/10, 1/100 and 1/1000, made up in a 20 mg/mL matrix solution of alpha-cyano-4-hydroxycinnamic acid (CHCA) in THF. 1000 shots were fired in sequences of 100 with the Smartbeam laser power at 90 % and summed to generate each spectrum. Mass spectra were recorded over a range of 3kDa-150Kda mass-to-charge ratio (m/z).

7.4.2. Phase transition studies on PNIPMAM: Using NanoDSF

LCST of the polymer was determined by NanoDSF. Different concentrations of PNIPMAM were prepared in dH₂O (0.1-1 wt%) and loaded into the instrument in capillary tubes. Temperature ramp rate was set to 0.4°C/min after scattering data was recorded from 20 – 75 °C. LCST was estimated by plotting first derivatives of the scattering intensity with the temperature change.

7.5. Experimental procedures from Chapter 3

7.5.1. Synthesis of IONPs

7.5.1.1. IONPs synthesized using polyol procedure (6 to 11 nm)

The synthetic preparation route was adopted from a previous study carried out by Maity *et al.*⁶ To synthesize 10 ± 2 nm IONPs, Fe(acac)₃ (0.7 g, 2 mmol) and 20 ml triethylene glycol (TREG) (Alfa Aesar) were added into in a three-neck round bottomed flask (RBF) equipped with condenser, magnetic stirrer, temperature probe and heating mantle. The reaction mixture

was heated at 120 °C for 1 h under argon to remove any moisture present in the system. After drying, the reaction mixture was heated to reflux (~280 °C) at a heating rate of 15 °C/min and kept at reflux for another 2 h. After the heating step, heating source was removed, and the reaction was cooled to room temperature. A black suspension of magnetite nanoparticles was obtained. Ethyl acetate was added (50 ml) to precipitate out NPs from the suspension. These black precipitates were separated magnetically using a neodymium magnet, re-dispersed in methanol (5 ml) and re-precipitated by adding excess ethyl acetate (50 ml). These washings were repeated 5 times. Washed NPs were dissolved in dH₂O and stored at 4 °C. Yield: 180 mg (78 %).

7.5.1.2. IONPs synthesized using thermal decomposition of Fe(acac)₃

7.5.1.2.1. Synthesis of 7 nm IONPs

Synthesis was adapted from a previous study by Sheng Tong *et al.*⁷ Fe(acac)₃ (1.4 g, 4 mmol), 1,2-teradecanediol (4.6 g, 20 mmol), OA (6.78 g, 24 mmol) and OAm (6.4 g, 24 mmol) were weighed in a three-neck round bottomed flask (RBF). Benzyl ether was then added (20.86 g) to the RBF and the reaction mixture was stirred at room temperature for 15 min prior to dehydration. The reaction mixture was then degassed at 100 °C for 1 h under vacuum along with continuous argon (Ar) purging. After degassing, the reaction temperature was raised to 200 °C at a heating rate of 5 °C/min and kept at this temperature for 2 h. The temperature was later increased to 300 °C (reflux) with the same rate for 1 h. After the heating step, heating source was removed, and the reaction was cooled to room temperature. The obtained black solution was then diluted in toluene (10 ml) and precipitated in ethanol (50 ml). The precipitates were separated magnetically (using a neodymium magnet) and washed 3 times with a mixture of toluene and ethanol (1:10). The washed precipitates were stored as a suspension in toluene (15 ml) at 4 °C. Yield: 260 mg (84 %).

7.5.1.2.2. Synthesis of 11 nm IONPs

Synthesis of monodisperse 11 nm IONPs was adapted from a previous study by Mohapatra *et al.*⁸ In a typical synthesis, Fe(acac)₃ (3.5 g, 10 mmol), OAm (8.0 g, 30 mmol) and OA (2.8 g, 10

mmol) were weighed in a three-neck RBF. The reaction mixture was stirred at room temperature for 15 min prior to the dehydration at 100 °C for 30 min with continuous Ar purging. After the dehydration step, the RBF was fitted with a condenser and the reaction mixture was heated to 150 °C under a positive Ar flow and kept at this temperature for 15 min. The temperature was then raised to 240 °C later at a heating rate of 5 °C/min for 4 h. The heating source was removed after 4 h and the reaction mixture was cooled at room temperature for 30 min. The obtained black solution was then diluted in toluene (10 ml) and precipitated in ethanol (50 ml). The precipitates were separated magnetically (using a neodymium magnet) and washed 3 times with a mixture of toluene and ethanol (1:10). The washed precipitates were stored as a suspension in toluene (30 ml) at 4 °C. Yield: 620 mg (81 %).

7.5.1.2.3. Synthesis of 16 nm IONPs

Synthesis of monodisperse 16 nm IONPs was adapted from a previous study by Mohapatra *et al.*⁸ In a typical synthesis, Fe(acac)₃ (3.5 g, 10 mmol), OAm (5.34 g, 20 mmol) and OA (2.8 g, 10 mmol) were weighed in a three-neck round bottomed flask (RBF). After the dehydration step, the RBF was fitted with a condenser and the reaction mixture was heated to 150 °C under a positive gas flow and kept at this temperature for 15 min. The temperature was then raised to 240 °C at a heating rate of 5 °C/min and kept at this temperature for 3 h. The temperature was later increased to 300 °C with the same heating rate and kept for 1 h. The heating source was removed after 4 h and the reaction mixture was cooled at room temperature for 30 min. Separation of the synthesized IONPs was done by similar method described for 11 nm IONPs synthesis. Yield: 580 mg (76 %).

7.5.1.2.4. Synthesis of 19 nm octahedrals IONPs

Synthesis of 18 nm nano-octahedrons was a modification of a previous study reported by Kim *et al.*⁹ Fe(acac)₃ (0.71 g, 4 mmol), OA (2.82 g, 10 mmol), 4-biphenyl carboxylic acid (0.793 g, 4 mmol) and dibenzyl ether (20.8 g) were weighed in a three-neck RBF fitted with a condenser and an over-head stirrer. The reaction mixture was stirred and degassed for 1 h by Ar purging. After degassing, the temperature was increased to 290 °C at a rate of 20 °C/min and stirred at

this temperature for 30 min. After heating, heating source was removed, and the reaction mixture was cooled down to room temperature. A mixture of hexane and toluene (1:1, 20 ml) was added and the black precipitates were separated by centrifugation. The precipitates were redispersed in chloroform and separated magnetically several times (3-5) and the final precipitates were dispersed in toluene (10 ml) and stored at 4 °C. Yield: 220 mg (71 %).

7.5.1.2.5. Synthesis of 27 nm IONPs

27 nm IONPs were prepared as described for 19 nm octahedral IONPs using Fe(acac)₃ (1.4 g, 4 mmol), OA (2.54 g, 9 mmol), 4-biphenyl carboxylic acid (0.793 g, 4 mmol) and benzyl ether (20.8 g). The synthesis was done under magnetic stirring instead using over-head stirrer to yield IONCs. Yield: 230 mg (75 %).

7.5.1.2.6. Synthesis of 35 nm iron oxide nanocubes (IONCs)

40 nm IONCs were synthesized by adapting a previous approach reported by Guardia *et. al.*¹⁰ Decanoic acid was used as shape specific ligand to synthesize cubic shaped NPs. Fe(acac)₃ (0.353 g, 1 mmol), decanoic acid (0.69 g, 4 mmol) and dibenzyl ether (25 ml) was added to a three-neck RBF. The reaction mixture was degassed at room temperature for 45 min and then heated to 200 °C with a rate of 5 °C/min for 2.5 h under Ar atmosphere. Reaction temperature was increased to 290 °C at a rate of 10 °C/min and kept at this temperature for 1 h. The reaction mixture was then cooled down to room temperature and acetone/chloroform (1:1, 100 ml) was added to precipitate synthesized nanocubes. The precipitates were separated by centrifugation and multiple washings of chloroform (3-5) were given to remove unreacted impurities. The final precipitates were dispersed in toluene (5 ml) and store at 4 °C. Yield: 65 mg (84 %).

7.5.2. Citrate coated water dispersible IONPs synthesis

Synthesis was adapted from a previous study⁸. For a typical synthesis, OA/OAm capped IONPs (100 mg) dispersed in toluene (10 ml) were sonicated for 30 minutes. Citric acid (5 mmol) dissolved in DMF (10 ml) was added to IONPs suspension and the mixture was sonicated for

another 30 min. The mixture was stirred for 6 h at 80 °C in a water bath. Citrate coated IONPs were separated magnetically from the reaction mixture, washed 5 times by methanol (20 ml) followed by magnetic separation to remove any unreacted citric acid. Citrate coated nanoparticles were then dispersed in dH₂O (5 mL) and stored in refrigerator (4 °C). Yield: 80 mg (80 %).

7.5.3. Iron oxide–PNIPMAM core-shell nanoparticle (IONP@PNIPMAM) synthesis

OA/OAm capped IONPs (30 mg, 1 ml of a 30 mg/ml suspension in toluene) and NDA-PNIPMAM (450 mg) were suspended in DMF (5 ml). The reaction mixture was sonicated for 5 h at room temperature, and left overnight stirring. The core-shell nanoparticles were then precipitated in diethyl ether (40 ml) and centrifuged (4500 rpm, 15 min). The supernatant was removed and the particles were re-dissolved in dH₂O (10 ml). The unreacted polymer was removed using ultracentrifugation (1600 g-force, 1 h at room temperature). The precipitates were dissolved in dH₂O (3 ml) and stored at 4 °C. Yield: 20 mg (~ 70 %).

7.5.4. Transmission electron microscopy (TEM)

TEM images were obtained using a JEOL 2011 transmission electron microscope operated at 200 kV accelerating voltage. CCD images were extracted using Gatan Digital Micrograph software. 1 mg/mL samples were prepared in water by sonication for 15 min and a drop of the dispersion was deposited onto 3 mm holey carbon coated copper grids. The grids were dried in air prior to analysis.

7.5.5. Determination of Iron content in the IONPs

Total iron content of IONPs was determined using UV-Vis spectrophotometer¹¹. The process was started with dissolving the known weight (5-10 mg) of IONPs in the minimum volume of conc. HCl (0.4 ml), resulting in the formation of a solution containing a mixture of Fe²⁺ and Fe³⁺ ions. The resulted solution was then diluted with dH₂O (25 ml) in a 50 ml volumetric flask and all the iron was then reduced to Fe²⁺ by adding excess of hydroxylamine hydrochloride (4 ml, 10 wt% in dH₂O). To this Fe²⁺ solution, o-phenanthroline (4 ml, 0.3 wt% in ethanol) was added

resulting in the formation of an orange red complex (pH = 6-6.5) and the content of the iron was determined using the UV-Vis spectrophotometer ($\lambda_{\text{max}} = 511 \text{ nm}$). The standard for the determination of the total iron content was Mohr salt.

7.5.6. Thermal gravimetric analysis (TGA)

TGA was carried out using 10 mg dry samples on a PL Thermal Sciences STA 625 instrument under air at a ramp rate of $10 \text{ }^{\circ}\text{C min}^{-1}$ between 0-600 $^{\circ}\text{C}$.

7.6. Experimental procedures from Chapter 4: Gel diffusion studies

7.6.1. Preparation of hydrogels

All the materials for making hydrogels were purchased from Sigma Aldrich unless mentioned otherwise. For agar, agarose, gelatin, pectin and carrageenan, a stock solution (0.5 wt%) was prepared by charging a sample vial with dH₂O (10 mL) and the appropriate quantity of powder (50 mg), then heating the vial to 70 $^{\circ}\text{C}$ to facilitate complete dissolution of the powder. 1 ml of this homogeneous solution was then poured to a small glass vial and left to gelate for 1 h at room temperature. Polyacrylamide gel was prepared according to the conditions reported in section 6.1.4. Poly(ethylene glycol)diacrylate (PEGDA) hydrogel was prepared according a literature method.¹² The hydrogel was prepared by charging 1 ml of 0.5 wt % PEGDA (in dH₂O) to a glass vial followed by 2 min of UV-radiation treatment to activate the cross linking reaction. The vial then left for 1 h at room temperature. The resulting gel vial had a volume of 2.0 mL and contained gel with a volume of 1.0 mL, a height of 0.018 m, and a diameter of 0.008 m.

7.7. Experimental procedures from Chapter 5: Proteins encapsulation and release studies

7.7.1. Proteins and antibodies

Proteins used in this study were purchased from Sigma Aldrich unless mentioned otherwise. Green fluorescent protein (GFP), apotransferrin (TRF) and bovine immunoglobulin (IgG) were

used as loading proteins. Ribonuclease A (RNaseA), ribonuclease B (RNaseB), ovalbumin (OVL), bovine serum albumin (BSA) and IgG were different competitor proteins used in this study. All antibodies except anti bovine IgG (Bethyl) were purchased from Bio Rad.

7.7.1.1. Preparation of protein-loaded nanoparticles

Prior to protein encapsulation, 0.5 mg of polymer-coated nanoparticles (100 μ L, 5 mg/ml PNIPMAM @ IONPs in dH₂O) were washed with diethyl ether (1 ml). Centrifugation (16,000 rpm, 20 min) was used to separate washed NPs. Supernatant was removed and the NPs were suspended in physiological buffer (100 μ L, 20 mM HEPES, 100 mM NaCl, pH 7.4). After ether washing, NPs were washed three times by suspending in physiological buffer (1 ml) followed by centrifugation. After washing, NPs were suspended in 1 μ g of loading protein (100 μ L of 1 g/l protein stock in physiological buffer). The solution was then incubated on a shaker at 45 °C for 10 min to agitate and suspend nanoparticles following precipitation at a temperature above polymer LCST (45 °C). NPs were then incubated on a shaker at room temperature for 2 h. Particles were then separated using centrifugation, and the supernatant retained as the unloaded protein sample. Particles were further washed by suspending them in 100 μ L of 10 mg/mL competitor protein solution in physiological buffer and incubating for 1 h at room temperature on a shaker. Particles were then separated using centrifugation and the supernatant was retained as first wash sample. This washing step was repeated multiple times (7 – 10), retaining each wash solution for further analysis. After washing, protein loaded NPs were stored in physiological buffer prior to further release experiments.

7.7.1.2. Protein release assays

0.5 mg of protein-loaded PNIPMAM @ IONPs were prepared and subjected to changes in temperature with or without magnetic heating. Experiments were conducted in 100 μ L solutions of 10 mg/mL competitor protein solution in physiological buffer. To study triggered protein release above polymer LCST without magnetic heating, NPs were suspended in the competitor protein solution and incubated at 45 °C for 1 h. During time points, NPs were regularly agitated and between time points, NPs were magnetically separated before removing 15 μ L of the sample volume for analysis. For magnetic heating experiment, pulsed

AMF (10 sec ON 30 sec OFF cycles) was used to specifically heat IONPs above LCST without considerable increase in bulk sample temperature. The samples were collected (15 μ l) and centrifuged to remove NPs. Supernatant was collected (10 μ l) and used for further analysis.

7.7.1.3. Sample preparation for protein analysis

Following collection, samples for apotransferrin were made up to 15 μ L in sample buffer (5 % (v/v) glycerol, 50 mM Tris-HCl pH 6.8, 50 mM dithiothreitol (DTT), 1 % (w/v) sodium dodecyl sulfate (SDS), 0.7 mM Bromophenol Blue) and boiled at 97 °C for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % (w/v) acrylamide gels prior to Coomassie staining or western blotting. For IgG samples, non-reducing sample buffer was used (buffer without DTT). IgG protein was separated by SDS-PAGE on 7 % (w/v) acrylamide gel and further analysed by western blotting.

7.7.1.4. SDS-PAGE

SDS-PAGE gels were prepared according to the conditions reported in table 7.1. For a separating gel composed of 10 % (w/v) acrylamide, 375 mM pH 8.8 tris buffer, 0.05 % (w/v) ammonium persulfate (APS) and 0.067 % (w/v) N,N,N',N'-tetramethylethylenediamine (TEMED), and a stacking gel containing 4 % (w/v) acrylamide, 125mM Tris pH 6.8, 0.1 % (w/v) APS, 0.1 % (w/v) TEMED. Samples were loaded into wells alongside 5 μ L of a pre-stained protein ladder (Precision Plus All-Blue, Bio-Rad). Gels were placed in gel tanks and immersed in running buffer (25 mM Tris, 250 mM glycine, 0.1 % (w/v) SDS) before running at 100 V constant voltage for 10 min and then at 150 V constant voltage until the dye had reached the bottom of the gel.

Table 7.1. Conditions to make different w/v (%) SDS-PAGE gels.

Percentage	Separating gel (w/v)				Stacking Gel 4 (w/v)
	7.5 (50-250 kDa)	10 (30-150 kDa)	12 (20-100 kDa)	15 (10-75 kDa)	

H ₂ O (ml)	6	5.1	4.2	3	2.7
Resolving Buffer (ml)	3	3	3	3	1.125
30 % Acrylamide (ml)	3	3.9	4.8	6	0.6
10% APS (μl)	100	100	100	100	40
TEMED (μl)	20	20	20	20	10

7.7.1.5. Coomassie staining

Fairbanks Coomassie staining was carried out by blocking gels in Fairbanks solution A (25 % (v/v) isopropanol, 10 % (v/v) acetic acid, 0.05 % (w/v) Coomassie brilliant blue) before washing with dH₂O. The process was repeated with Fairbanks solution B (10 % (v/v) isopropanol, 10% (v/v) acetic acid, 0.005 % (w/v) Coomassie brilliant blue), Fairbanks solution C (10 % (v/v) acetic acid, 0.002 % (w/v) Coomassie brilliant blue) and Fairbanks solution D (10 % (v/v) acetic acid). Gels were left in solution D until distinct bands were apparent from the background. Quantification was carried out using ImageJ software.

7.7.1.6. Western blotting

Western blotting was conducted by semi-dry transfer of gels onto nitrocellulose membranes (Thermo Fisher) for 70 min at 0.3A using 48 mM Tris-HCl, 39 mM glycine, 20 % (v/v) MeOH and 0.0375 % (w/v) SDS as the transfer buffer. After membrane transfer, different blocking procedures were used for apotransferrin and IgG. For apotransferrin, membranes were blocked using phosphate buffered saline (PBS) with 0.05 % (v/v) Tween-20 (PBST) and 5 % (w/v) milk for 1 h at room temperature. Membranes were then incubated with primary antibody: antiapotransferrin (1:500, Dako) in 5 % (w/v) milk PBST solution overnight at 4 °C. Following six 10 min washes at room temperature in 5 % (w/v) milk PBST solution, secondary antibody goat anti rabbit-horseradish peroxidase (1:1000, Bio-Rad) in 5 % (w/v) milk PBST solution was added for 1 h at room temperature. The blot was then washed 3 times with 5 %

(w/v) milk PBST solution (5 min each) and 3 times in PBST for 10 min each at room temperature. For IgG, membranes were blocked in tris-buffered saline (TBS) with 0.05 % (v/v) Tween-20 (TBST) and Roche blocking solution (1:10) for 1 h at room temperature. Membranes were then incubated with goat anti bovine IgG-horseradish peroxidase (1:250) for 1 h followed by three 5 min washings with blocking solution followed by three 10 min TBST washings. Blots were imaged on Invitrogen iBright imaging systems after application of Immobilon horseradish peroxidase (HRP) substrate (Millipore). Quantification was carried out using ImageJ software.

7.8. Reference

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8. Commonly used abbreviations

AMF = Alternating current magnetic field

ATRP = Atom transfer radical polymerisation

brm = Broad multiplet

CDCl_3 = Deuterated chloroform

Da = Dalton

D_2O = Deuterated water

DCM = Dichloromethane

dH_2O = Deionised water

DIPEA = *N, N*-diisopropylethylamine

DLS = Dynamic light scattering

DMF = Dimethylformamide

$\text{DMSO-}d_6$ = Deuterated dimethyl sulfoxide

DOX = Doxorubicin

h = Hour(s)

HBr = Hydrogen bromide

HCl = Hydrochloric acid

IONPs = Iron oxide nanoparticles

IONOs = Iron oxide nano-octanes

IONCs = Iron oxide nanocubes

IPA = Isopropylamine

LCST = Lower critical solution temperature

MeCN = Acetonitrile

m/z = Mass-to-charge ratio

MALDI-TOF = Matrix-assisted laser desorption/ionisation-time of flight

M_n = Number average molar mass

MRI = Magnetic resonance imaging

M_w = Weight average molar mass

mM = Millimolar (concentration)

nm = Nanometre

mg = Milligram(s)
mmol = Millimole(s)
kHz = Kilohertz
mL = Millilitre(s)
μL = Microlitre(s)
min = Minute(s)
ng = Nanogram
NPs = Nanoparticles
NaOH = Sodium hydroxide
NMR = Nuclear magnetic resonance
NDA = 6-Nitrodopamine hydrogensulfate
NDA-PNIPMAM = 6-Nitrodopamine hydrogensulfate-terminated poly(*N*-isopropylmethacrylamide)
NIPMAM = *N*-isopropylmethacrylamide
PNIPMAM = Poly(*N*-isopropylmethacrylamide)
PNIPMAM @ IONPs = poly(*N*-isopropylmethacrylamide) coated iron oxide nanoparticles
PDI = Polydispersity index
ppm = Parts per million
RBF = Round bottomed flask
SAR = Specific absorption rate
TEM = Transmission electron microscopy
TRF = apotransferrin
UCST = Upper critical solution temperature
W/g = Watts per gram
XRD = X-Ray diffraction

9. Appendices

Appendix A

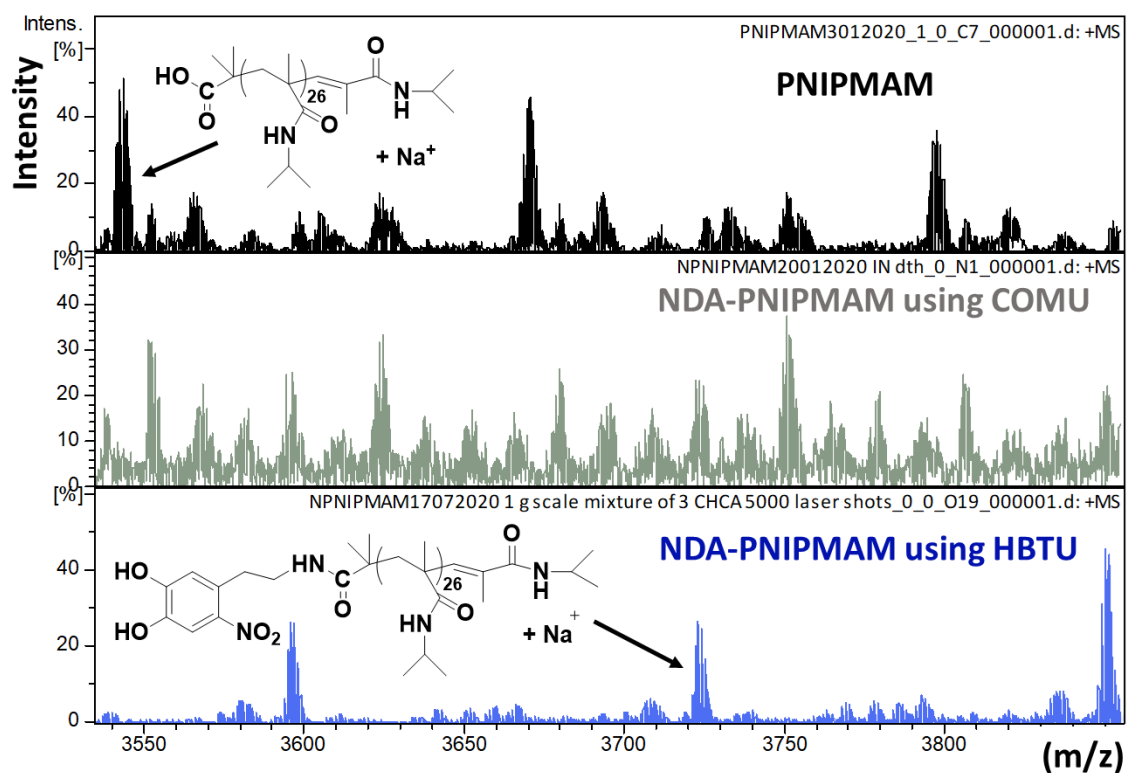


Fig. 9.1. MALDI-MS spectra of PNIPMAM, and NDA-PNIPMAM synthesized using COMU and HBTU (coupling agent).

Appendix B

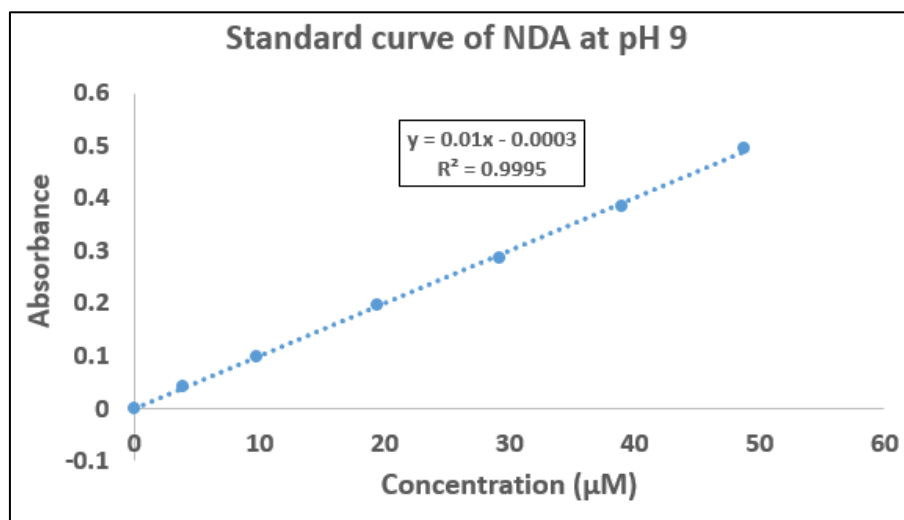


Fig. 9.2. Standard curve of NDA at pH 9 used to calculate the molar extinction coefficient of NDA = $9600 \text{ mol}^{-1}\text{cm}^{-1}$.

Appendix C

Polymer shell thickness on PNIPMAM @ IONPs

Polymer shell thickness on PNIPMAM @ IONP was estimated theoretically by taking the bulk density of polymer as the high limit density of a polymer-coated NP. For a non-planar NP surface, the volume (V) of the polymer on the NP ($V(\text{polymer})$) can be calculated as:

$$V(\text{polymer}) = V(\text{NP with polymer}) - V(\text{NP without polymer}) \quad (9.1)$$

For a spherical polymer coated NP, equation can be rewritten as:

$$V(\text{polymer}) = \frac{4}{3} \pi [(R_P^3 - R_{NP}^3)] \quad (9.2)$$

Here, R_P and R_{NP} are the NP radius with and without polymer (TEM), respectively. $V(\text{polymer})$ can also be written as equation 9.3.

$$V(\text{polymer}) = \frac{\text{Mass of a polymer chain}}{\text{Density of the polymer}} \quad (9.3)$$

Where,

$$\text{Mass of a polymer chain} = \frac{M_w \text{ of the polymer}}{N_A} \quad (9.4)$$

Here, N_A (Avogadro's constant) = $6.022 \times 10^{23} \text{ mol}^{-1}$. The density of PNIPAM in water is known (1.1 gcm^{-3} , 30°C)^{24,25} which is mainly the density of water (1 gcm^{-3}). Since PNIPAM and PNIPMAM only differ in one extra methyl group their repeat unit, they will have similar density in water below the LCST. Hence, Density of PNIPMAM used = $1 \times 10^{-21} \text{ gnm}^{-3}$. Polymer shell thickness of different 40 kDa PNIPMAM @ IONP is then calculated using equation 8.2. For 40 kDa PNIPMAM @ $15.4 \pm 2.1 \text{ nm}$ IONP, the polymer shell thickness was calculated as $\sim 2.5 \text{ nm}$ and hence the diameter of core-shell NP would be $\sim 20.4 \text{ nm}$.

Appendix D

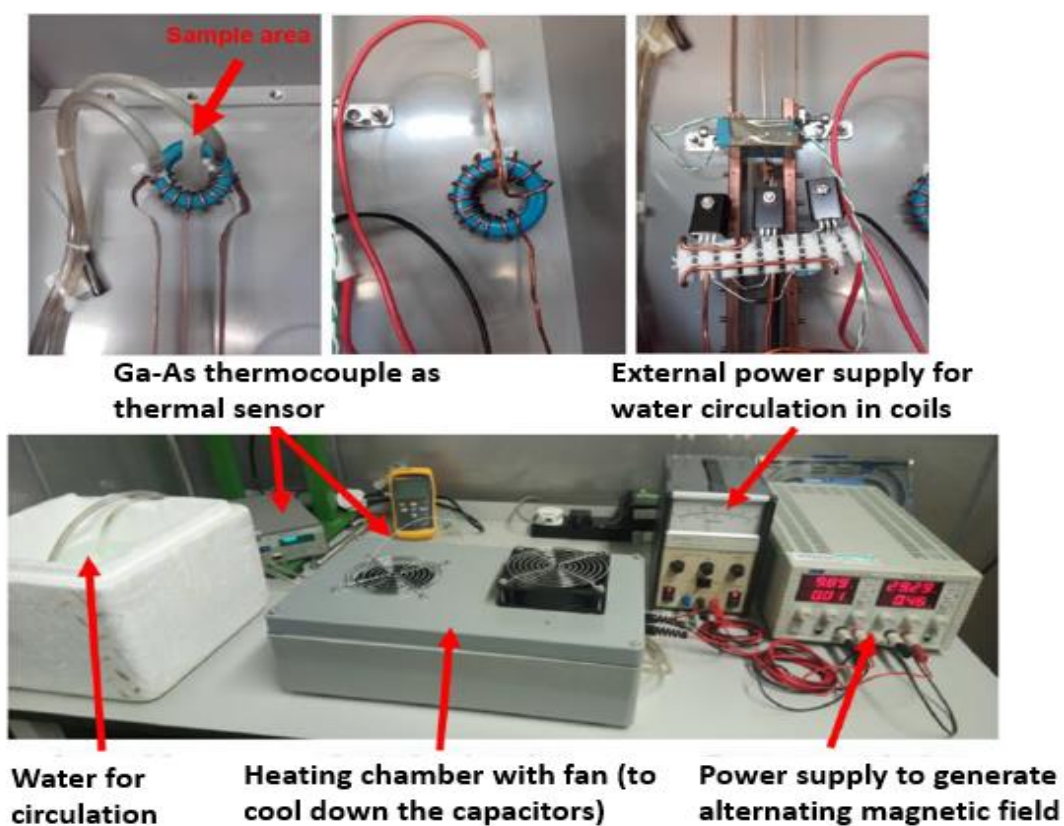


Fig. 9.3. Magnetic heating setup used for SAR measurements of IONPs.

Appendix E

Estimation of the mesh size of 0.5 wt% agar hydrogel

Gel mesh size of 0.5 wt% agar hydrogel was calculated by using elastic blob theory, which is appropriate for the swollen networks. This model assumes that the polymers are well-described as elastically effective chains and that the gel mesh size is equal to the size of the elastic blob (ξ) given by equation 9.5.

$$\xi = P_{el}^{-1/3} \quad (9.5)$$

where P_{el} is equal to the number density of elastic blobs and is calculated from the zero-frequency shear modulus (G_0') using equation 9.6.

$$G_0' = P_{el} k_B T \quad (9.6)$$

where k_B is Boltzmann's constant ($1.38 \times 10^{-23} \text{ m}^2 \text{ Kg s}^{-2} \text{ K}^{-1}$) and T is temperature (298 K). G_0' for 0.5 wt% agar hydrogel at 298 K was $\sim 150 \text{ Pa}$ ($1 \text{ Pa} = 1 \text{ Kg m}^{-1} \text{ s}^{-2}$). Hence, the mesh size of the hydrogel was calculated using equation 9 and 10 as 30.2 nm.

Appendix F

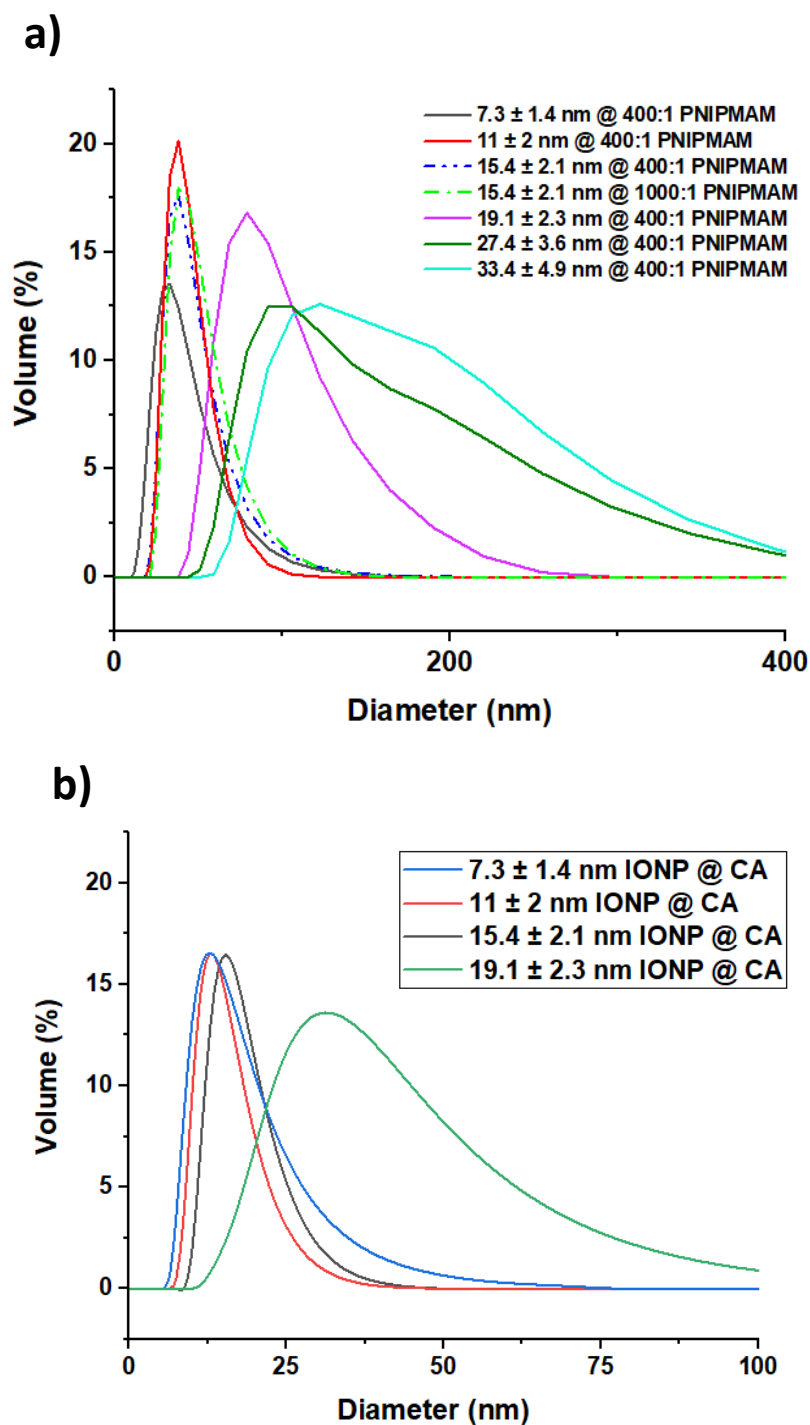


Fig. 9.4. DLS spectra of IONPs: a) various PNIPMAM @ IONPs. Here, 400:1 and 1000:1 PNIPMAM represents 40 kDa and 89 kDa PNIPMAM, respectively. b) DLS spectra of various CA @ IONPs.

Appendix G

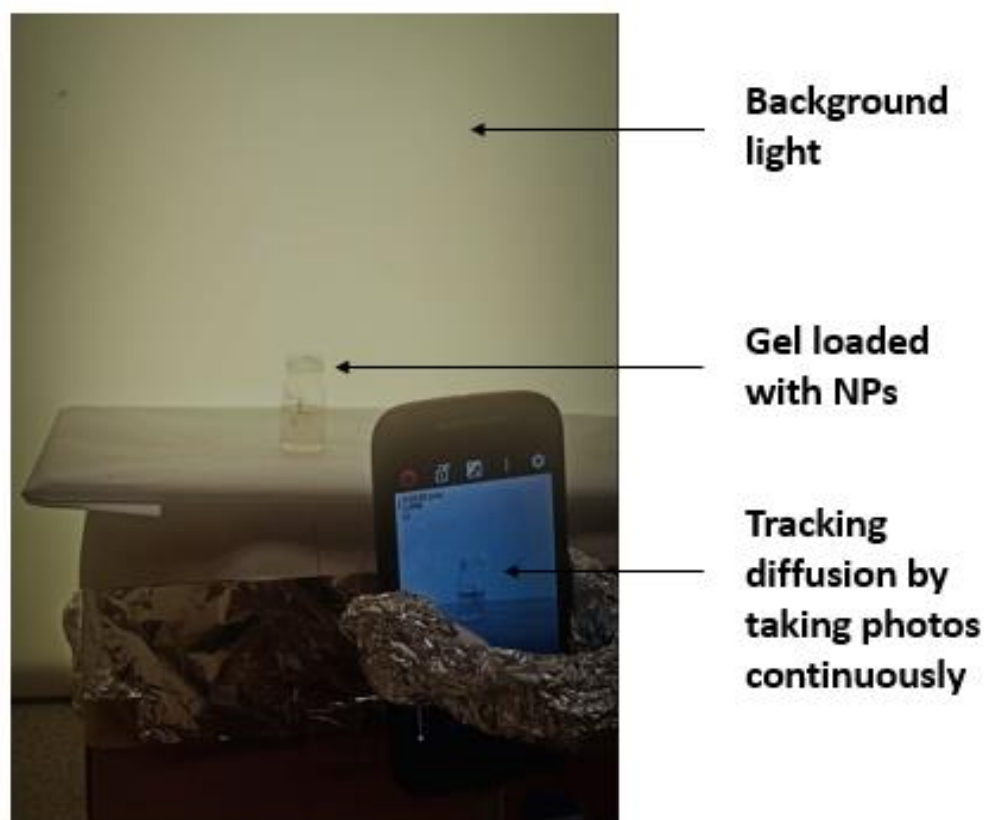


Fig. 9.5. Setup used to monitor the migration of aqueous NPs through hydrogel (Dark box not shown).

Appendix H

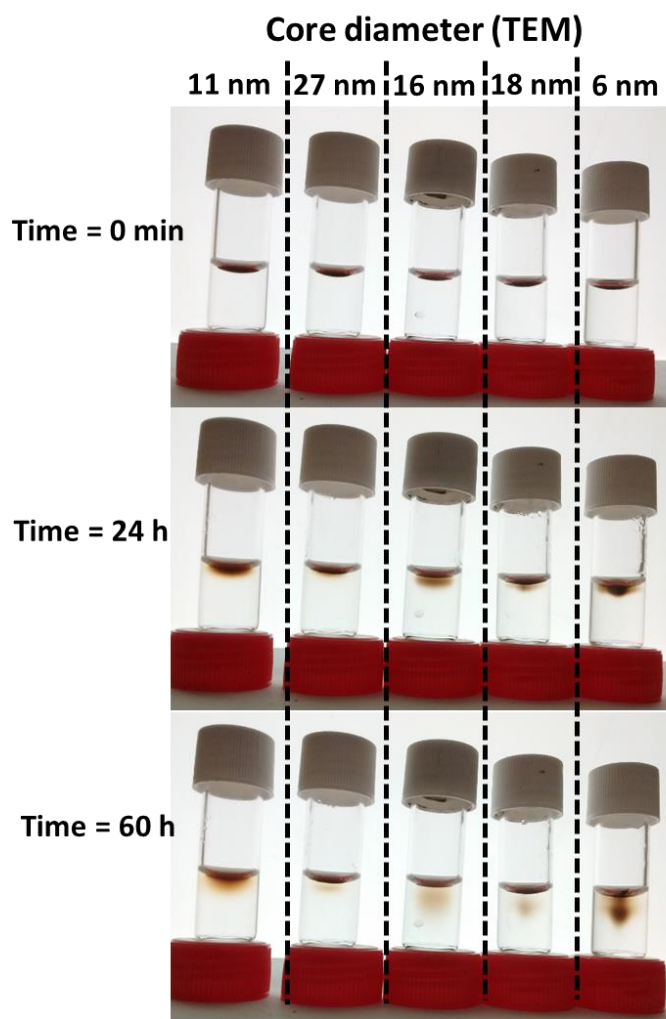
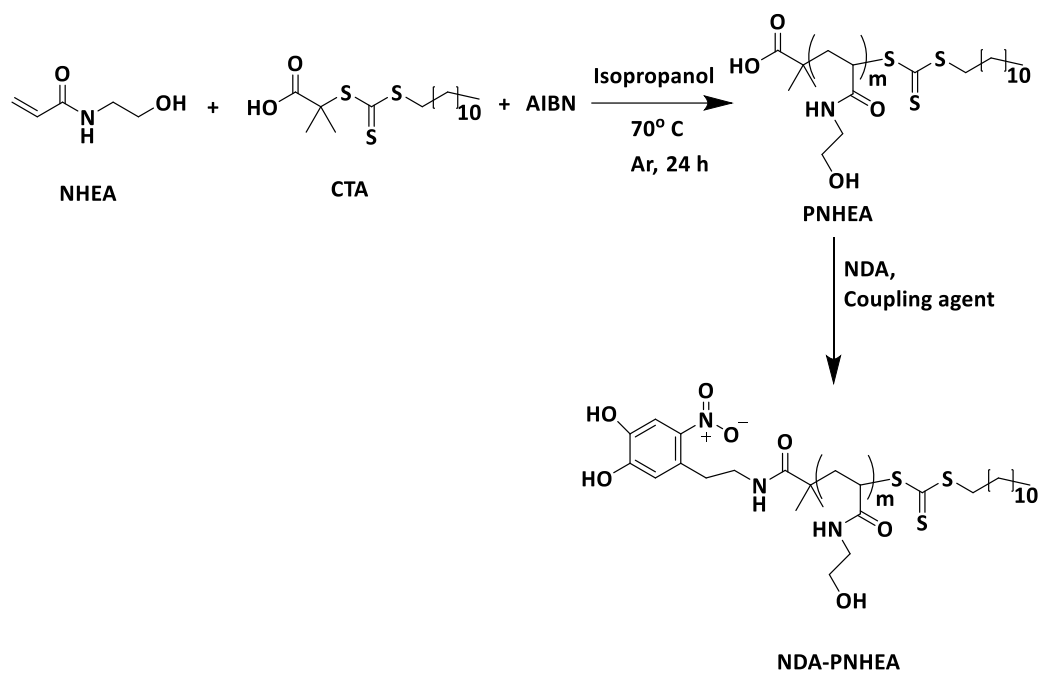


Fig. 9.6. Images at different time intervals from the gel diffusion experiment of 40 kDa PNIPMAM @ IONPs (6 nm, 11 nm, 16 nm, 19 nm and 27 nm core diameter). [NP] = 5 mg/ml of [Fe].



Fig. 9.7. Measuring position of the nanoparticle front at different times in a gel diffusion experiment of NPs using Gel_Diffusion2.exe software.

Appendix I



Scheme. 9.1. RAFT polymerization and end group modification to give NDA end group PNHEA. Polymerization scale: NHEA: CTA: AIBN = 400:1:0.2. Average molecular weight ($^1\text{H-NMR}$) = 19 kDa (**appendix J**).

Appendix J

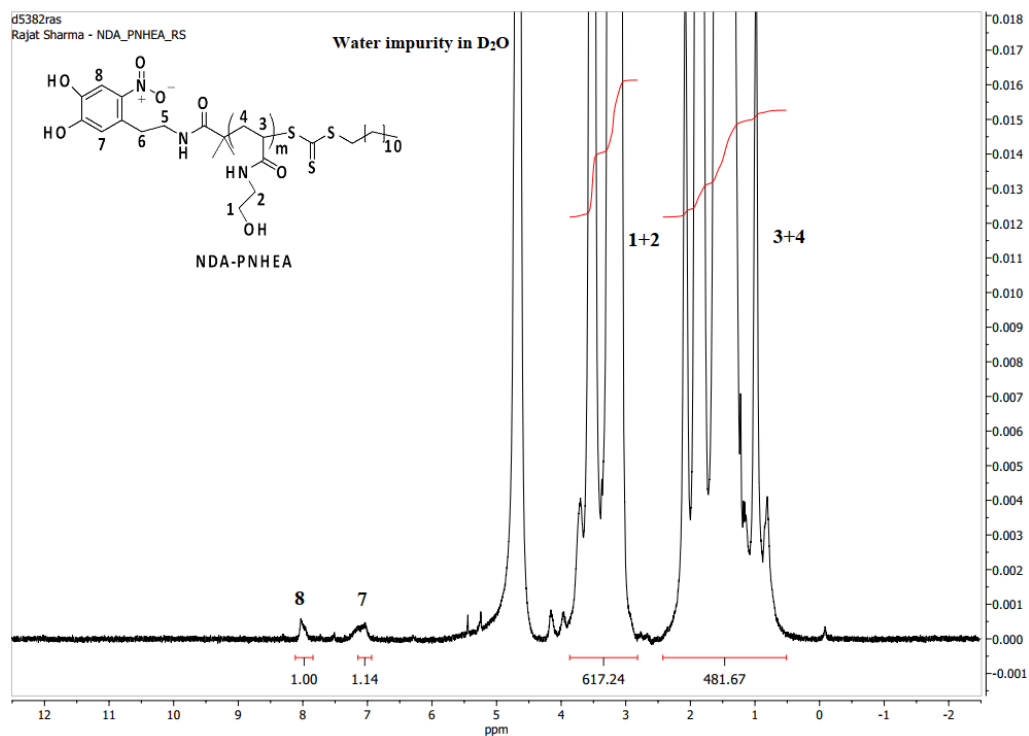


Fig. 9.8. ^1H -NMR of NDA-PNHEA in D_2O . Average molecular mass (M_w) was calculated by integrating nitrodopamine functionalities of PNHEA with respect to PNHEA protons (1+2). Mass of a repeat unit = 115 g/mol. M_w is calculated as = $115 \times 617.24/4 = 17,745$ Da.

Appendix K

Represented blots for the TRF loading/release with

different molecular weight PNIPMAM

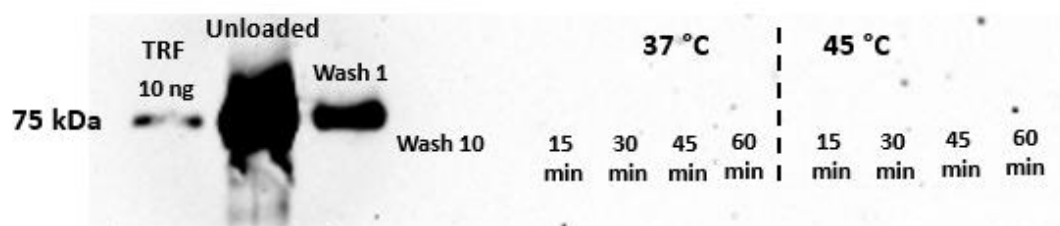


Fig. 9.9. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 7.5 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30, 45 and 60 min at 37 °C and 45°C.

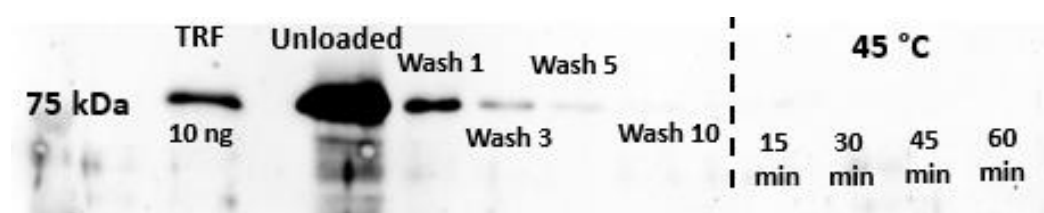


Fig. 9.10. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 19 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30, 45 and 60 min at 45 °C.

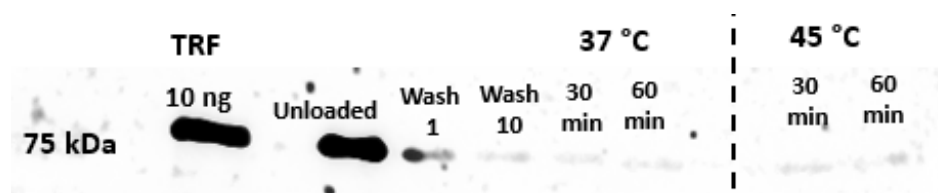


Fig. 9.11. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 27 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

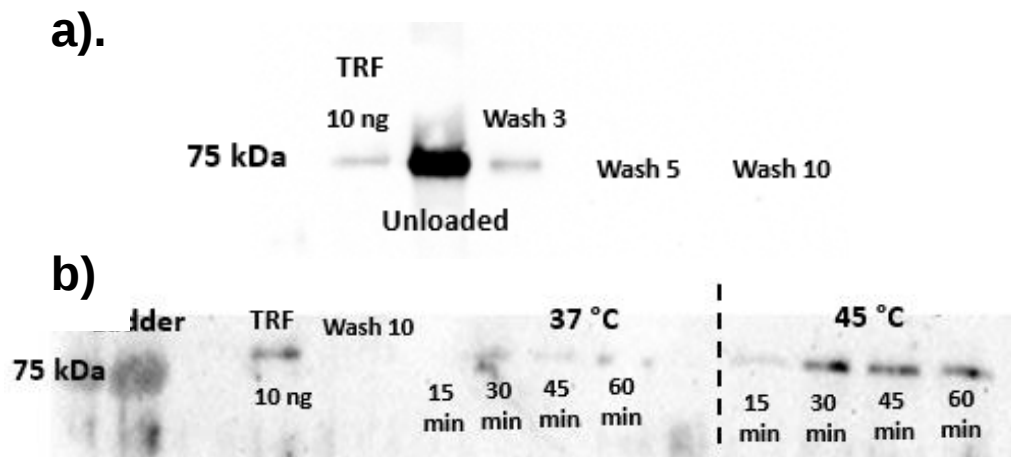


Fig. 9.12. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4. **a)** Unloaded TRF and washing samples, **b)** protein release samples after the incubation of washed TRF-loaded NPs for 15, 30, 45 and 60 min at 37 °C and 45 °C.

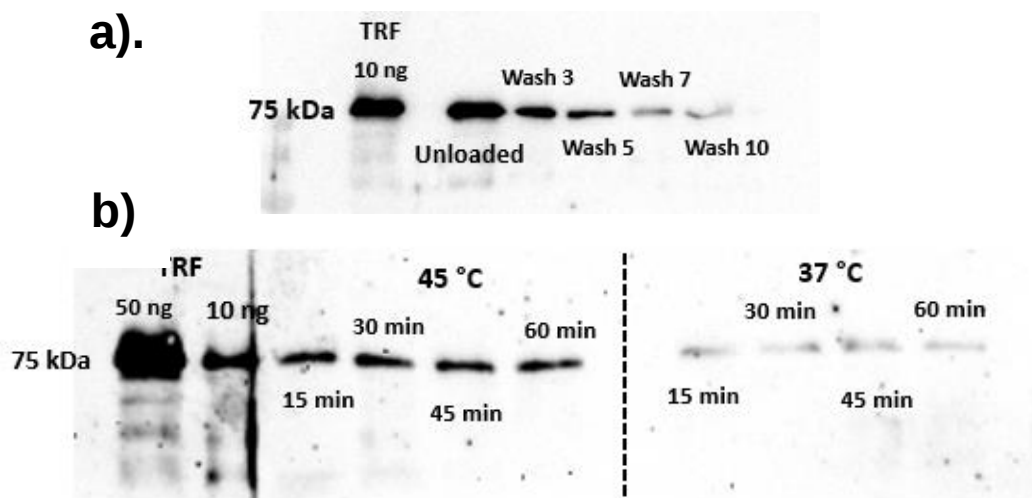


Fig. 9.13. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 89 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4. **a)** Unloaded TRF and washing samples, **b)** protein release samples after the incubation of washed TRF-loaded NPs for 15, 30, 45 and 60 min at 37 °C and 45 °C.

Appendix L

Represented blots for the TRF loading/release with different core-size IONPs

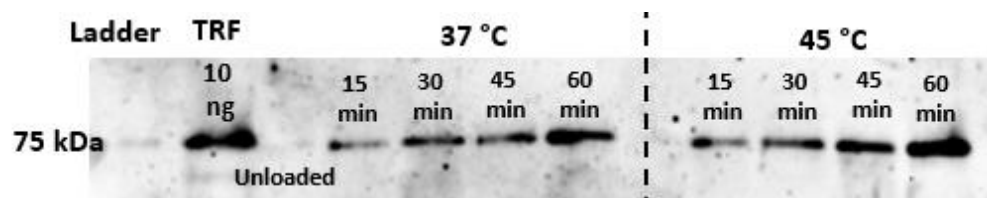


Fig. 9.14. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 40 kDa PNIPMAM @ 7 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30, 45 and 60 min at 37 °C and 45 °C.

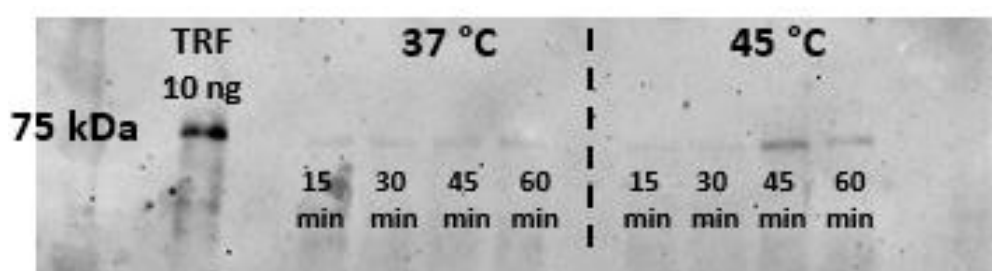


Fig. 9.15. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 40 kDa PNIPMAM @ 11 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30, 45 and 60 min at 37 °C and 45 °C.

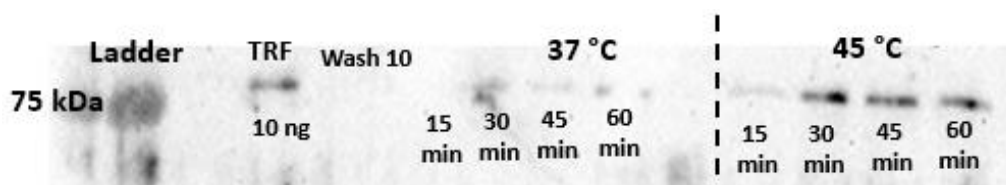


Fig. 9.16. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 40 kDa PNIPMAM @ 11 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30, 45 and 60 min at 37 °C and 45 °C.

Appendix M

TRF release with different competitor proteins

Glycosylated proteins – OVL and IgG:

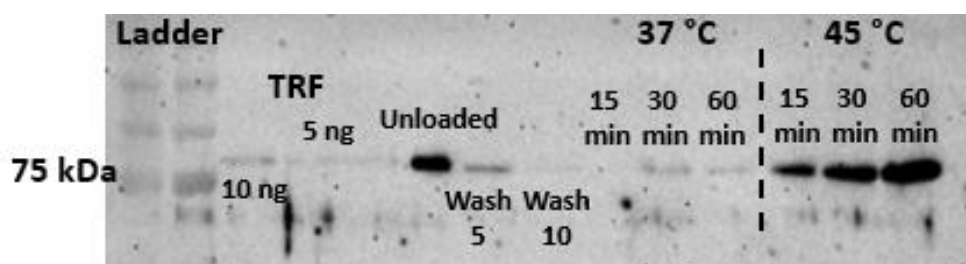


Fig. 9.17. Western blot analysis following 10 mg/ml OVL treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30 and 60 min at 37 °C and 45 °C.



Fig. 9.18. Western blot analysis following 10 mg/ml IgG treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 15, 30 and 60 min at 37 °C and 45 °C.

Non-glycosylated proteins- RNaseA and BSA:

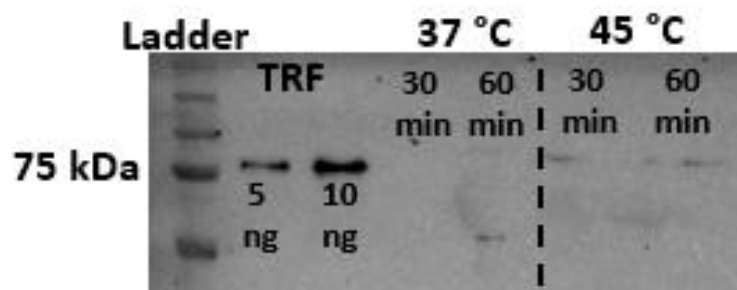


Fig. 9.19. Western blot analysis following 10 mg/ml RNaseA treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

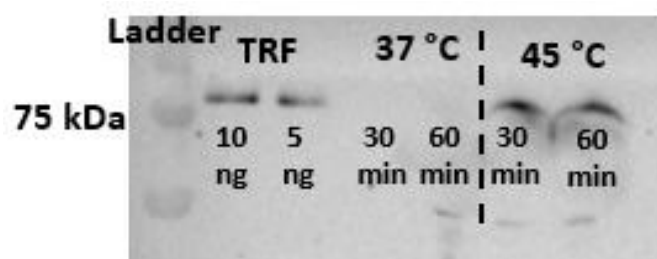


Fig. 9.20. Western blot analysis following 10 mg/ml BSA treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g TRF at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

Appendix N

IgG release with different competitors

Glycosylated proteins – RNaseB, OVL and TRF:

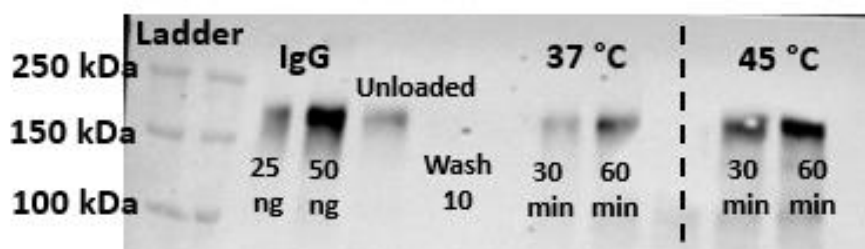


Fig. 9.21. Western blot analysis following 10 mg/ml RNaseB treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

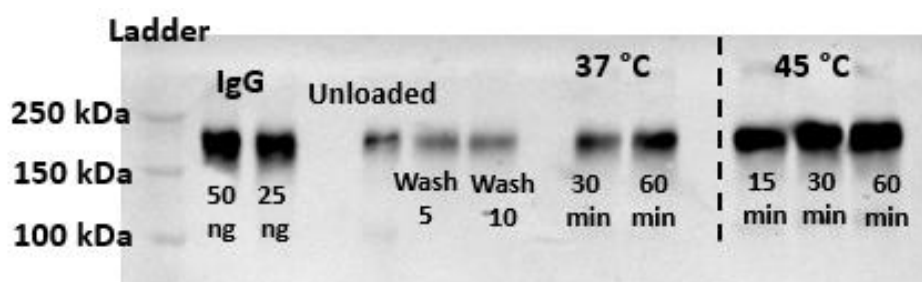


Fig. 9.22. Western blot analysis following 10 mg/ml OVL treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

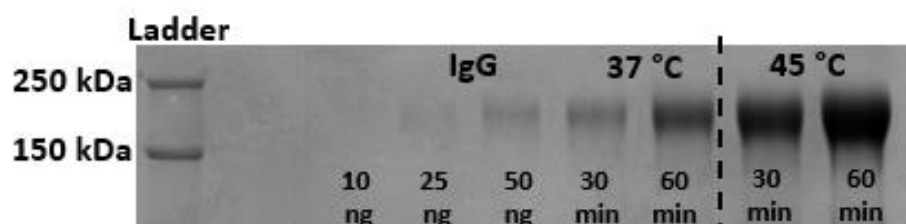


Fig. 9.23. Western blot analysis following 10 mg/ml TRF treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

Non-glycosylated proteins – RNaseA and BSA:

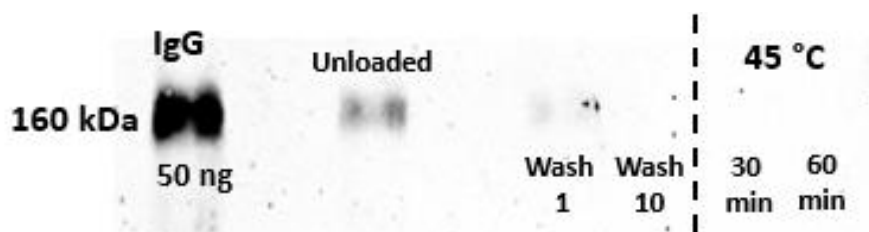


Fig. 9.24. Western blot analysis following 10 mg/ml RNaseA treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 45 °C.

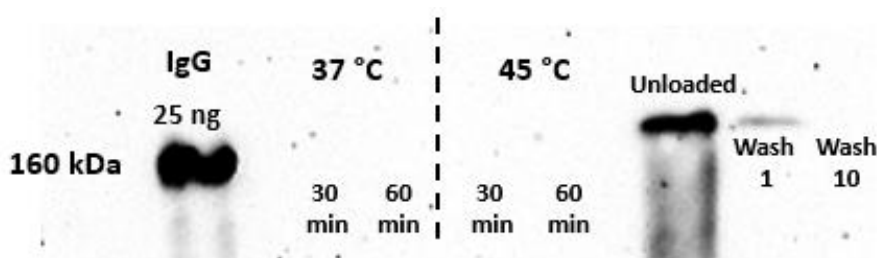


Fig. 9.25. Western blot analysis following 10 mg/ml BSA treatment of 0.5 mg 40 kDa PNIPMAM @ 16 nm IONPs incubated with 1 μ g IgG at pH 7.4 for 30 and 60 min at 37 °C and 45 °C.

Appendix O

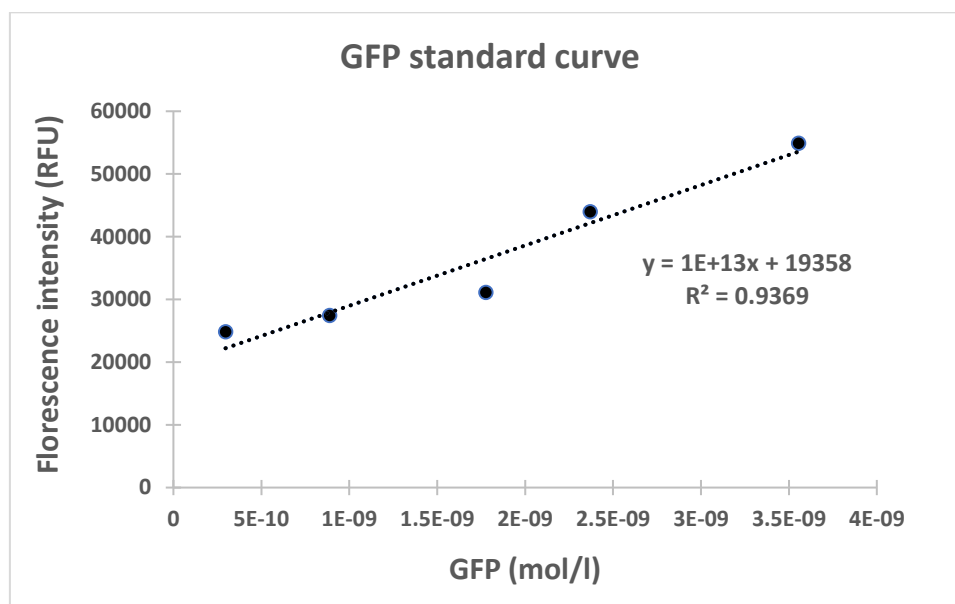


Fig. 9.26. Standard GFP curve used for the analysis of GFP loading/release samples.