NANOTECHNOLOGY ENHANCED IMMUNOASSAY FOR PREECLEMPSIA

by

Syeda Fatima A. Zaidi

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Committee:

Dr. Alessandra Luchini, Thesis Director

Dr. Lance Liotta, Committee Member

Dr. Robin Couch, Committee Member

Dr. Iosif Vaisman, Acting Director,
School of Systems Biology

Dr. Richard Diecchio, Interim Associate
Dean for Student and Academic Affairs,
College of Science

Dr. Peggy Agouris, Interim Dean,
College of Science

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Manassas, VA

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A thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at George Mason University

By

Syeda Fatima A. Zaidi
Masters in Pharmaceutical Chemistry
University College of Pharmacy, 2009
Bachelors of Pharmacy
University College of Pharmacy, 2005

Director: Alessandra Luchini, Assistant Professor
College of Science

Spring Semester 2014
George Mason University
Manassas, VA

DEDICATION

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A**BSTRACT**

NANOTECHNOLOGY ENHANCED IMMUNOASSAY FOR PREECLAMPSIA

Syeda Fatima A Zaidi, MS

George Mason University, 2013

Thesis Director: Dr. Alessandra Luchini

N-isopropylacrylamide (NIPAm)-based, bait-loaded hydrogel particles are successfully used to capture, sequester and protect from enzymatic degradation low abundance, low molecular weight labile proteins in body fluids. In current protocols, proteins captured by the nanoparticles are eluted with chemical buffers and analyzed with immunoassays or mass spectrometry techniques. Here, a novel, *in situ* ELISA is presented in which captured proteins are probed with a labeling antibody directly inside the nanoparticles without the need of chemical elution. Dually crosslinked (degradable N,N'-(1,2-Dih ydroxyethylene)- bisacrylamide [DHEA] and nondegradable N,N′-methylenebis(acrylamide) BIS] crosslinkers) NIPAm particles functionalized with trypan blue were used. DHEA can be degraded by an oxidizing agent (e.g. NaIO4) thus increasing the pore size of the hydrogel nanoparticles and allowing the antibody to access the antigen previously trapped in the nanoparticle. Interleukin 6 (IL6) was chosen as a

model molecule to test the nanoparticle based ELISA. IL-6 is a protein associated with preeclampsia, an autoimmune, hypertensive pregnancy-related medical problem that can lead to serious complications that can be life threatening to mother and child.

Dually crosslinked NIPAm particles efficiently sequestered IL6 from human urine. Oxidative degradation of DHEA crosslinker by $NaIO_4$, caused a partial erosion of the DHEA crosslinker resulting in loose network of hydrogel polymer and increased access of antibody to the captured IL6 protein. Captured IL6 was preserved from oxidative degradation by virtue of the interaction between the protein and trypan blue chemical bait. The IL6 ELISA, based on these dually cross linked NIPAm particles, showed a limit of detection of 600 picograms and two orders of magnitude dynamic range. Coefficient of variations was constantly below 5%. Sensitivity of the test can be improved by increasing the volume of urine processed. It is anticipated that this technology can be successfully used to detect true positive preeclamptic patients from a group of gestational hypertensive population.

CHAPTER 1

INTRODUCTION

Nanotechnology has recently been exploited as an efficient enabler in in-vitro diagnostics (IVD) (1). Early detection improves therapeutic outcome as well as management of many medical complications (2). The use of biological fluids such as urine or blood to gather molecular insights in disease pathogenesis and early detection of disease is highly desirable in current and future medical practice. Nevertheless, research and development of molecular diagnostics is impeded by the following unresolved issues:

1. Disease biomarkers exist in body fluid at very low concentration (below the detection limit of current diagnostics and discovery technologies
2. Disease biomarker are rapidly degraded by endogenous and exogenous enzymes immediately after sample procurement
3. Disease biomarkers can be masked by high abundance resident proteins such as albumin and immunoglobulin.(3)

Recently, capturing nanoparticles were developed to address abovementioned unresolved issues (4). Bait functionalized harvesting nanoparticles have the following functions: 1) size sieving, 2) protection from degradation, 3) sequestration and enrichment. (5)

Captured protein analytes are eluted by chemical means and subjected to immunoassay and mass spectrometry analysis (6, 7). Here, we propose the synthesis of novel, partially degradable harvesting nanoparticles and the application of these particles to a one step, *in situ* immunoassay. Partially degradable nanoparticles are mixed with the biofluid of interest and in minutes capture the target analytes. A brief (few minutes) oxidizing treatment cleaves the degradable cross linkers and increases the pore size of the nanoparticles. This in turn allows selected antibody to penetrate the interior area of the nanoparticles and successfully interact with the protein antigen trapped inside. In this way, the nanoparticles trap the target analyte in solution and provide support for the labeling antibody. Partially degradable nanoparticles were obtained by combining degradable cross linker N,N'-(dihydroxyethylene)bisacrylamide (DHEA) and non degradable crosslinker N,N'methylenebisacrylamide (BIS) with thermo responsive polymer N-isopropylacrylamide (NIPAm). These dually cross linkedNIPAm particles were functionalized with acrylic acid and trypan blue as bait. Following is a step-wise description of harvesting dual croslinked particle based immunoassay

1. Closely crosslinked nanoparticles (degradable and non degradable crosslinkers) are incubated with the solution of interest and harvest and preserve all the solution phase target analyte
2. Nanoparticles are separated from the solution and washed. Degradable crosslinkers are broken by NaIO4. This partial degradation of the particles results in increase in pore size as well as increase in diameter of the treated particles.

3. Degraded nanoparticles containing target analytes are probed with horseradish peroxidase (HRP)-labeled monoclonal antibodies raised against the target analyte. Degraded nanoparticles present increased pore size that allows monoclonal antibodies to interact with the captured analyte.
4. Chromogenic HRP substrate is added to the nanoparticle-antibody complex and colorimetric reaction is measured with a spectrophotometric ELISA plate reader;

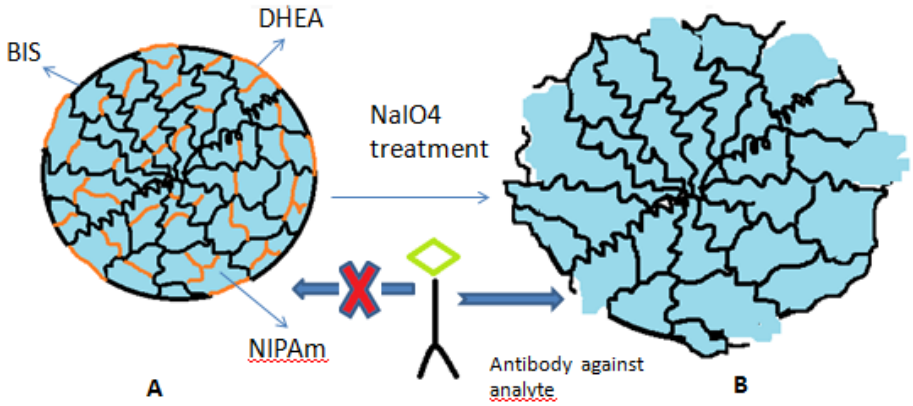


Fig 1.1. A Dually crosslinked particle presents small pore size. B Degradation of DHEA by NaIO4 results in larger pore size and increase in diameter.

These particles were used to detect IL-6 as an early predictor of preeclampsia. It is anticipated that the developed test will allow to detect true positive preeclamptic patients (increased levels of IL-6 in early pragnancy) from a getational hypertensive population and help diagnosis of preeclampsia before the appearance of clinical symptoms.

CHAPTER 2

POLYMER CHEMISTRY

2.1. Hydrogels

Hydrogels are defined as loose polymeric network that can swell in water. On the basis of rheology, hydrogels are defined as cross-linked polymers that exhibit viscoelastic or pure elastic behavior. The swelling capacity of hydrogels is enormous; they can absorb water about thousand times more than their dry weight(8). This water-absorbing property of the hydrogels has made them useful for contact lenses(9), drug delivery(10), protein separation(11), tissue engineering and catalysis.(12)

2.2. Hydrogels classification

Hydrogels can be classified in many ways but the most important classification is based on the type of cross-links. Cross-links are important to maintain the ‘network’ structure of the hydrogels and to prevent dissolution of the hydrophilic chains. Based on the type of cross-links there are two classes of hydrogels(13): physically cross-linked and chemically crosslinked hydrogels. Hydrogels can also be classified based on their size as macrogels or microgels. Hydrogels can be

further classified as stimuli-responsive or non-responsive gels. Non-responsive gels, as the name suggests, are materials that swell upon water absorption. On the other hand stimuli-responsive gels are considered to be “smart” materials because they respond to subtle changes in the environment. These hydrogels can be made responsive to temperature(114), pH(15), ionic strength(16), light, electric field (17)and biomolecules(18). The “smart” behavior of the hydrogels is inherited from the type of the polymer that is used in making these gels.(19)

2.3. Poly (N-isopropylacrylamide) (PNIPAm)

Due to its thermo-sensitive behavior in aqueous solutions, pNIPAm has been widely used to make responsive hydrogels.

- **Thermo-sensitive Behavior of PNIPAm**

  The behavior of a polymer in a solvent is a balance of solvent-solvent interactions, solvent-polymer interactions and polymer-polymer interactions. In aqueous enrironments, hydrogen bonds form between pNIPAm amide side chains and water molecules . water molecules form ordered structures around the isopropyl group. In aqueous solution pNIPAm has a random coil structure because the solvent-polymer interactions are stronger than the polymer-polymer interactions. At higher temperatures the hydrogen bonds with water molecules break and there is an entropically-favored release of water. In this case the

polymer-polymer interactions become stronger than the polymer-solvent interactions and the polymer phase separates.

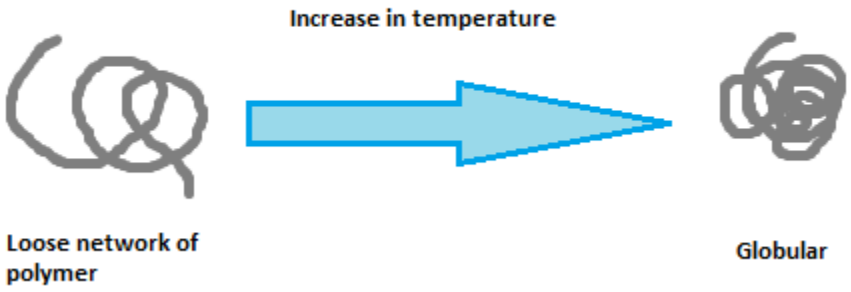


Fig.2.1. Effect of temperature on NIPAm polymer chain

The temperature at which this phase separation occurs is called the lower critical solution temperature (LCST). Figure 2.1 shows the coil to globule transition of the pNIPAm polymer chain. This behavior of pNIPAm makes it a very attractive candidate for making stimuli-responsive hydrogels. The LCST behavior of pNIPAm has been studied by a variety of techniques: UV-VIS, differential scanning calorimetry, light scattering, viscometry and fluorescence. (20)

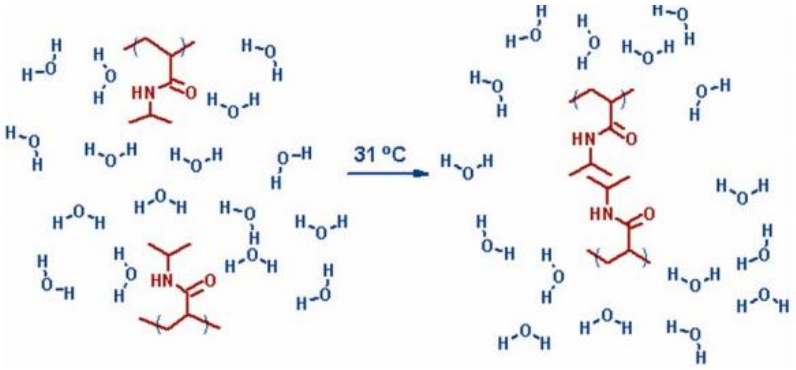


Fig. 2.2: Effect of LCST on NIPAm. Adapted from Synthesis and Characterization of Multiresponsive Microgels, Satish P. Nayak(20)

2.4. Cross linker.

The formation of bonds which hold portions of several polymer chains together is called cross-linking. Cross-linking results in a random three-dimensional network of interconnected chains. As one might expect, extensive cross-linking produces a structure which has more rigidity, hardness, and a higher melting point than the equivalent polymer without cross-linking**.**

- **N,N' methylenebisacrylamide (BIS).**

  BIS is a non degradable cross linker. It has a great effect on the pores size of thermosensitive particles. Increase in BIS concentration reduces effective pore size while increasing the network heterogeneity(21).

- **N,N'-(dihydroxyethylene)bisacrylamide (DHEA).**

  DHEA is a degradable cross linker that presents a 1,2 diol moiety between two acrylamide groups. The carbon - carbon bond between two alcohol groups can be cleaved by periodate ion.

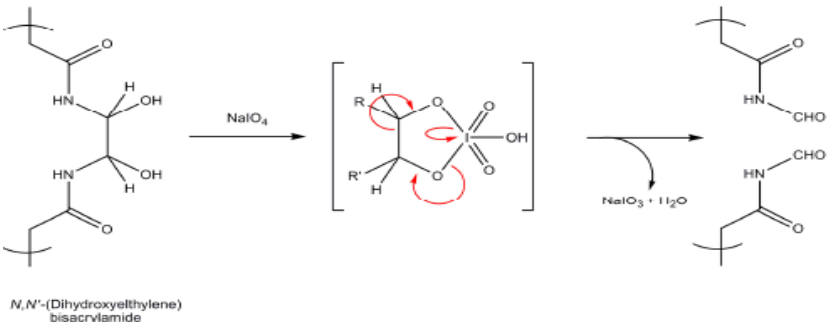


Fig. 2.3: DHEA degradation by NaIO4. Adapted from Synthesis and Characterization of Multiresponsive Microgels, Satish P. Nayak(20)

DHEA has been used in different nanostructures modulating their specialized structure and function such as hollow shell and core particles (22)

2.5.Co-monomer: Acrylic Acid (AAc)

Different synthetic high molecular weight polymers of acrylic acid (AAc) are being used in biomedical applications. They may be homopolymers of acrylic acid, crosslinked with an allyl ether pentaerythritol, allyl ether of sucrose or allyl ether of propylene (23). In a water solution at neutral pH, poly acrylic acid

(PAAc) is an anionic polymer, i.e. many of the side chains of PAAc will lose their protons and acquire a negative charge. This makes PAAc polyelectrolytes, with the ability to absorb and retain water and swell to many times their original volume (24)

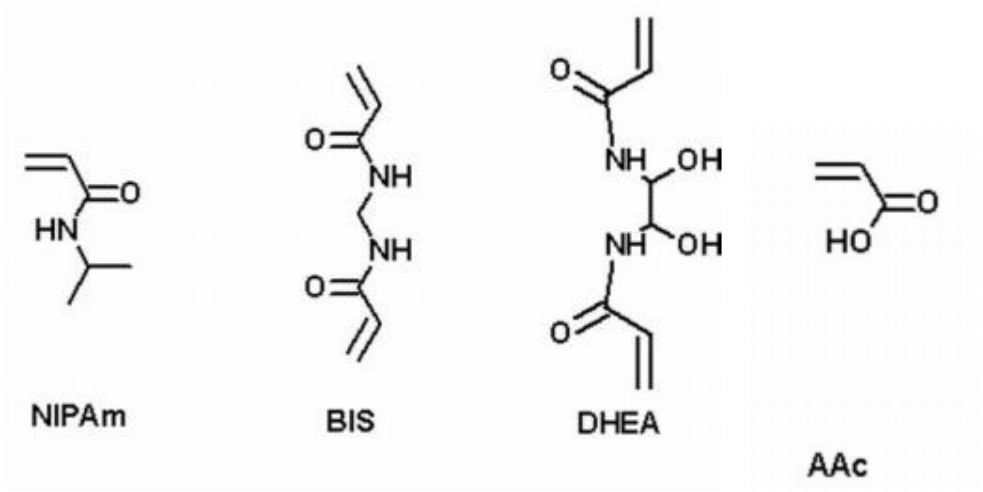


Fig.2.4: Common monomers used in the synthesis of nano particles. NIPAm – N-isopropylacrylamide BIS – N,N'- methylenebisacrylamide DHEA – N,N'-(1,2-Dihydroxyethylene)-bisacrylamide AAc – Acrylic acid.

2.6. Polymerization reactions

There are different types of reactions for thermosensitive hydrogel synthesis such as photo polymerization(25), dispersion polymerization(26), free radical precipitation polymerization(27) chain-transfer polymerization(28). The choice of reaction depends on the reactants and desired type of product polymer.

- **Free radical precipitation polymerization**

  In this study, we used free radical precipitation polymerization. Free radical precipitation polymerization is a heterogeneous polymerization process that begins initially as a homogeneous system in the continuous phase, where the monomer and initiator are completely soluble. Upon initiation, the growing polymer is insoluble and thus precipitates. After precipitation, the polymerization proceeds by absorption of monomer and initiator into the polymer particles.(29)

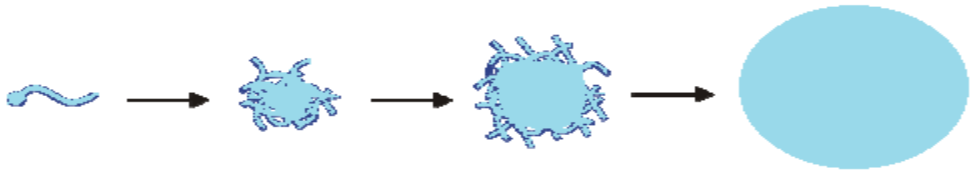


Fig. 2.5: Synthesis of NIPAm microgel through free radical precipitation polymerization.

2.7. Characterization of Microgels

Several techniques are used to characterize microgels including such as light scattering, differential scanning calorimetry, fluorometry, small-angle neutron scattering, UV-VIS spectrophotometry, rheology and NMR.(30) In our lab dynamic light scattering (DLS) was used to study the solution behavior of the microgels.(31)

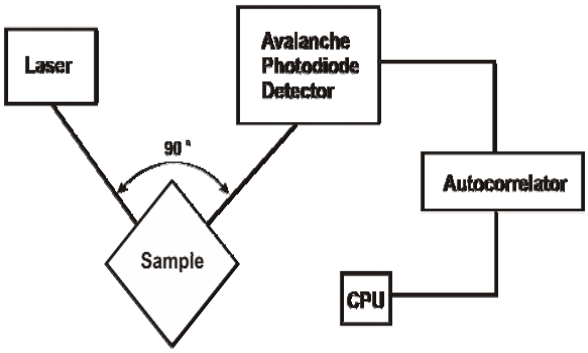


Fig.2.6: A: Schematic of Dynamic Light Scattering (DLS) setup. Adapted from Design, Synthesis and Characterization of Multiresponsive Microgels, Satish P. Nayak (20)

The light sheds on the sample through fiber optics and the scattered light at 90° to the source is collected by an avalanche photodiode. The signal from the photodiode is fed to an autocorrelator board, from which the diffusion coefficient is calculated. In DLS, the fluctuations in the scattered intensity are collected and these fluctuations are used to plot

the autocorrelation function. The diffusion coefficient is then calculated from the decay of the autocorrelation function. Assuming that the particles have random Brownian motion the diffusion coefficient is used to calculate the hydrodynamic radius (Rh) of the particles from the Stoke-Einstein equation

$$R_h = \frac{k_b T}{6\pi\eta D}$$

Where kb is the Boltzman constant, T is the solution temperature in Kelvin, η is the solvent viscosity and D is diffusion coefficient. (32)

2.8 Applications of Microgels

Microgels have potential applications in several fields including diagnostics(31), drug delivery(33), biosensing(34), chemical separations(35), catalysis (36)and optics (37)

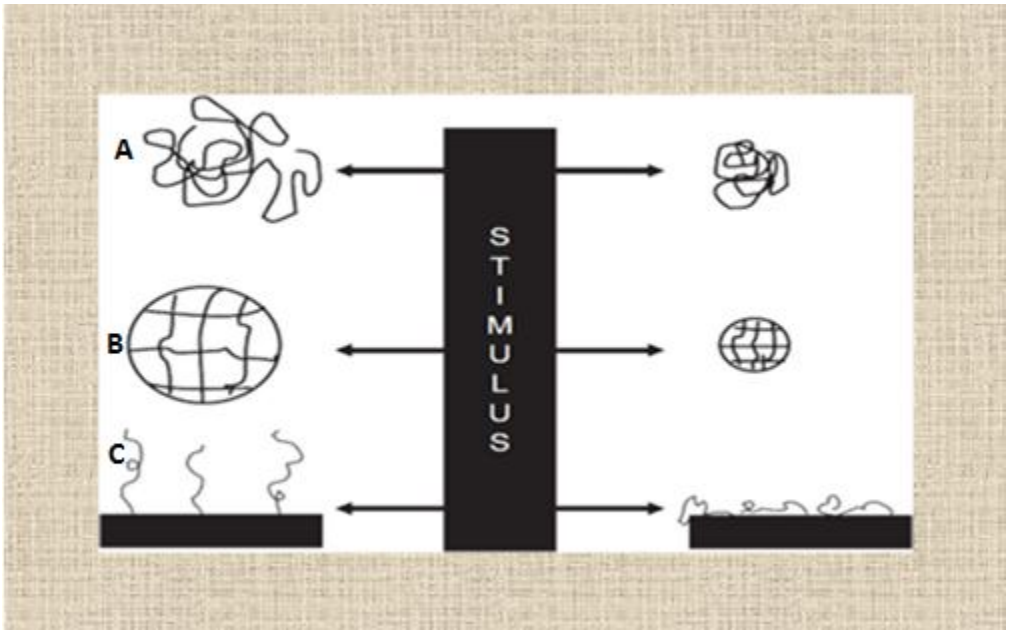


Fig. 2.7: A Linear free chains in solution, B covalently cross-linked reversible and physical gels and C chains adsorbed or surface-grafted forms. After stimulus, A Collapse of linear free chains B Swelling or shrinking of a gel C Swelling or collapsing on surface. Adapted from Design, Synthesis and Characterization of Multiresponsive Microgels, Satish P. Nayak(20)

CHAPTER 3

PREECLAMPSIA

International Society for the Study of Hypertension in Pregnancy (ISSHP) has defined pre-eclampsia as de -novo hypertension (systolic blood pressure [BP] ≥140 mmHg or diastolic BP ≥90 mmHg) after gestational week 20 plus proteinuria (>0.3 g/24 hours).(38) Other signs and symptoms include edema and headache, and, in severe cases, the condition is associated with seizures (eclampsia), liver and kidney dysfunction, clotting abnormalities, Adult Respiratory Distress Syndrome and fetal growth restriction. Potential determinants of pre-eclampsia include an array of risk and protective factors, including familial factors, maternal smoking, pre-existing medical conditions (such as hypertension, diabetes mellitus and anti-phospholipid syndrome), and miscellaneous ones such as plurality, older maternal age and obesity. Hypertensive disorders are associated with elevated liver enzymes and low platelets syndrome, severe morbidity, especially in cases of severe pre-eclampsia, eclampsia and haemolysis, and higher rates of maternal, fetal and infant mortality. (38)

Preeclampsia affects 6-8% of all pregnancies worldwide (39). In fact, it is estimated that the actual incidence of preeclampsia is higher than the official estimates because of inaccurate assessment and lack of diagnosis of the conditions.

Early prediction of preeclampsia is highly desirable because of the following reasons:

- Preeclampsia's subtle symptoms share so many commonalities with other conditions present during normal pregnancy that a diagnosis can be delayed or missed(40)
- Use of conventional sphygmomanometry devices/ automated oscillometric devices are subject to significant inter- and intra-observer error leading to underdiagnosis(41)
- The development of proteinuria is often a late sign in pre-eclampsia and a poor predictor of disease severity and progression
- Maternal biochemical abnormalities can also occur as a result of end organ damage, but their utility as predictors of pre-eclampsia is limited.(42)
- Current literature on a preeclampsia test is inconclusive. Use of varying definitions for early- and late-onset disease and disease severity has made direct comparisons difficult.(43)
- Data relating to pre-pregnancy screening for pre-eclampsia are limited.(44)
- The cause of preeclampsia remains unknown. However, the placenta is involved, because preeclampsia can occur in molar pregnancies when placental tissue is present and fetal tissue is absent; the delivery of the placenta is the only known

cure for preeclampsia. Because pre-eclampsia is a disease of placental dysfunction, placental biomarkers are generally altered earlier in gestation, before maternal manifestations of the disease

- Finally, preeclampsia is a serious pregnancy related complication and continues to be a major threat to the maternal and prenatal lives (45)

3.1. Proteins with diagnostic potential for preeclampsia:

A specific and sensitive diagnostic test should detect a protein or a panel of proteins associated to the earliest event in the pathogenesis of the disease. Preeclampsia is a disease of placenta, so it will be valuable to investigate biological events during early placentation in order to select most specific predictive protein indicators.

- **Biology of placentation.**

Exact biology of implantation is still unknown but it has been proposed as a highly orchestrated phenomenon involving various immunological, metabolic, invasive, angiogenic steps. (46) During human implantation and placentation, the invasion of a subset of fetal trophoblast cells, called extravillous trophoblast, into the maternal uterus enables the attachment of the placenta to the uterine wall and the transformation of maternal spiral arteries to facilitate adequate nutrition of the fetus. Till delivery, maternal and fetal factors closely interact to maintain pregnancy and smooth the process of delivery. (47) Each gestational stage is strictly controlled by the immune system. It has been proven that placentation starts with a slight immunological reaction on the basis of discrimination between ‘self’ and ‘non-self’. Accordingly, the fetus activates the

mother's immune system because the fetus is in part 'non-self'. (48) This reaction creates a localized inflammation followed by hypoxia. In hypoxic environment, maternal and fetal interfaces start invasion. At some point T_{reg} cells dampen the immunological reaction and smooth the process of delivery. So, a successful pregnancy depends on constraints on maternal immunity. Preeclampsia is an outcome of lost constraints. Therefore, preeclampsia stems from immunological rejection of fetus by maternal immune system and is an auto immune disorder.

Histological examination of placental bed biopsies from preeclamptic women demonstrates trophoblast proliferation but limited migration into superficial decidua. Consequently, invasion of the cells into the myometrial portions of the spiral arteries is severely reduced, resulting in reduced intervillous blood flow and placing the fetus at risk of oxygen and nutrient deprivation. Villous trophoblasts from preeclamptic placentae have been found to exhibit an immature phenotype, ultrastructurally and biochemically, when compared with normal placentae. (49) The shallowly implanted placenta which becomes hypoxic is thought to stem from immunological rejection of fetus by maternal immune system and causes preeclampsia.

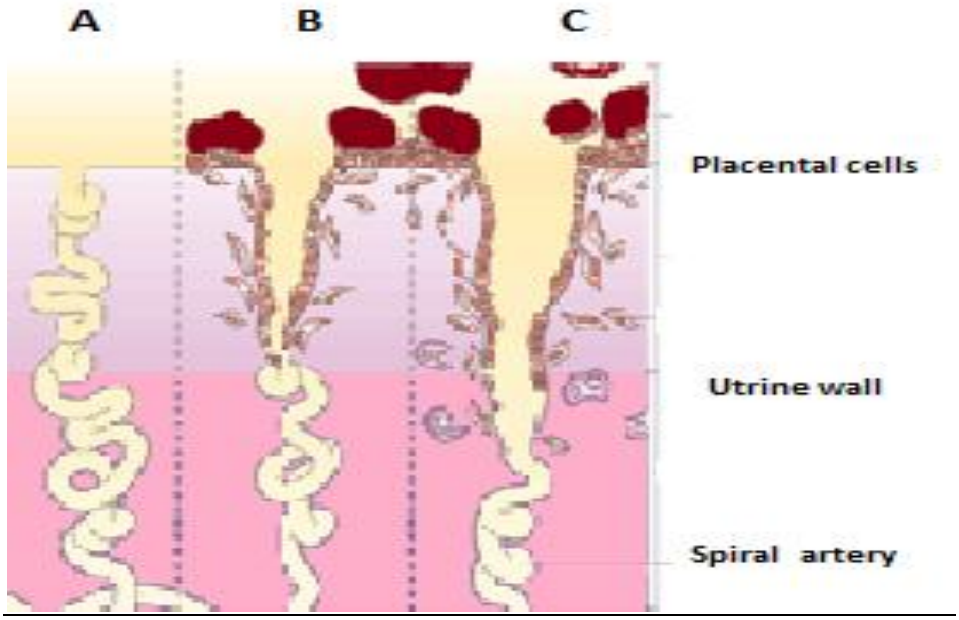


Fig 3.1: A. Non-pregnant uterine spiral artery, Abnormal cytotrophoblastic invasion in B. pre-eclampsia and C, normal pregnancy. Modified from nature reviews, Immunology **4**, 927 (December 2004)

- **IL-6 induces Th17/ Treg imbalance.**

It has been shown that Th17/ Treg imbalance causes the onset of various autoimmune and chronic inflammatory diseases. IL-6, in combination with TGF-β, promotes the differentiation of naïve T cells into Th17 IL-17-producing T helper cells, but inhibits TGF-β-induced Treg differentiation. Dysregulated IL-6 production leads to predominance of Th17 over Treg. The resultant Th17/Treg imbalance leads to breakage of immunological tolerance and is of pathological importance for the development of various autoimmune and chronic inflammatory. (50)

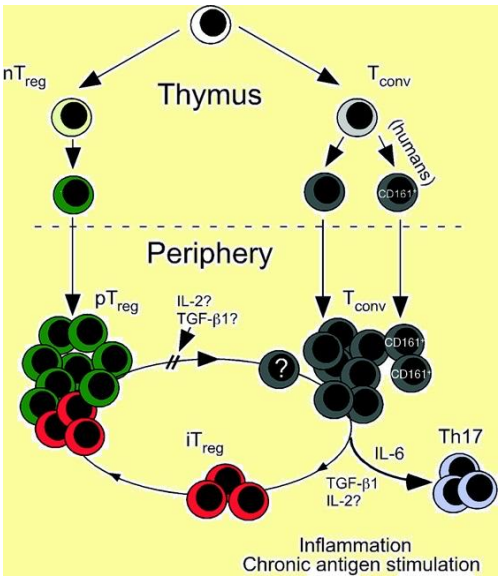


Fig 3.2: Induced Treg (iTreg) cells and Th17 cells are derived from a common naïve conventional T (Tconv) precursor population. Th17 pathway is favored in the presence of IL-6. Both iTreg and Th17 cells require TGFβ1 for their development. Adapted from. The Treg/Th17 Cell Balance: A New Paradigm for Autoimmunity Pediatric Research (2009) 65

The above explanation for the shift in Th17/Treg balance towards Th17, an effector cell for the induction of autoimmune diseases and the key role of IL-6 in the imbalance, suggests IL-6 as a valuable protein analyte for diagnostic test for preeclampsia. There are many clinical studies that present evidence of a correlation between preeclampsia, autoimmune diseases and role of IL-6. (51)

It has been found that women with systemic lupus erythematosus (SLE) have a two-fold increase in risk of preeclampsia. Women with Rheumatoid arthritis have a two-fold increase of this severe pregnancy complication. Preeclampsia risk was greater in women with SLE compared to women without an autoimmune disease. Some autoimmune diseases, such as arthritis, are temporarily ameliorated, and regulatory T cells have been shown to be responsible for this beneficial effect. Unfortunately, the diseases return with a vengeance after delivery and do not seem to benefit from the generation of protective regulatory T-cell memory. (52) Above mentioned contrasting evidences imply that Treg specificity is very important in regulating the balance between immune activation that maintains host defense and immune suppression that prevents autoimmunity. So, the conditions in which T regulatory cells sufficiently suppress immune response, result in improvement of existing autoimmune symptoms (at least during pregnancy) and vice versa. IL-6 is the responsible factor for the inhibition of TGF β induced Treg differentiation as well as increase in Th17 cell maturation. IL-6 has a pleiotropic effect but its abnormal persistent production causes the onset and development of various autoimmune and chronic inflammatory diseases. (53)

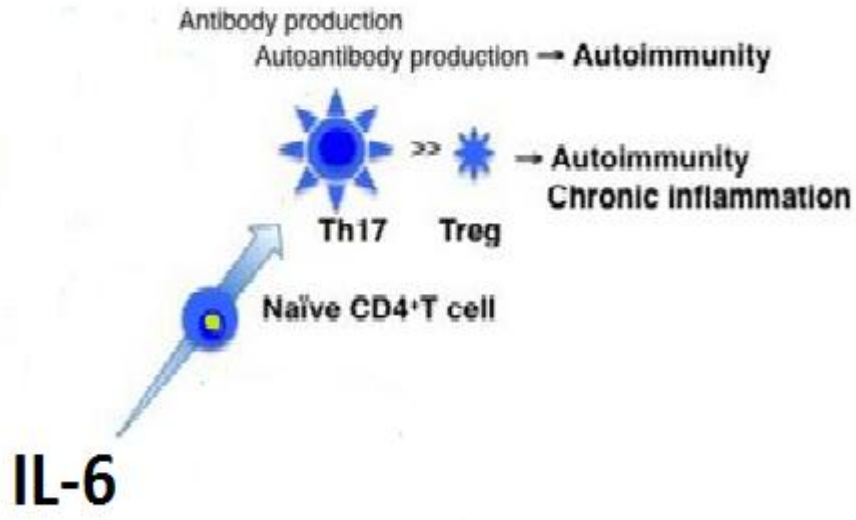


Fig 3.3: Role of IL-6 in autoimmunity. Adapted from Targeting Interleukin-6: All the Way to Treat Autoimmune and Inflammatory Diseases, Int J Biol Sci 2012 (54)

A humanized anti-human IL-6R monoclonal antibody (chemical name: tocilizumab) was used to treat rheumatoid arthritis as a monotherapy or in combination with disease-modifying antirheumatic drugs.(54) Ttocilizumab significantly suppressed disease activity and radiographically detected progression of joint deformity, thus improving daily functional activity. (55)

Above stated facts reinforce the specificity of IL-6 for autoimmune events. Many clinical studies showed that IL-6 is present in higher concentration in women with preeclampsia than in normal patients (56)

CHAPTER 4

MATERIALS AND METHODS

4.1.Nanoparticle synthesis

NIPAm based particles were synthesized with the following molar percentages:

4.1.1 88% NIPAm/ 12% DHEA

4.1.2 77% NIPAm/ 8% BIS / 8% DHEA / 7%AAc

For each reaction following quality controls were set:

- Homogenous suspension
- Adequate sedimentation after centrifugation
- Appropriate particle size (300-1000nm)

4.1.1. 88% NIPAm 12% DHEA particles synthesis

N-Isopropylacrylamide (NIPAm, Sigma-Aldrich, 712mg, 6.2 m mol) and 1,2 – Dihydroxyethylene bis-acrylamide (DHEA, Sigma-Aldrich 169mg, 0.845mmol) were dissolved in 95 mL of H_2O, filtered using a nitrocellulose membrane disk filter (0.45 μm pore size, Millipore), and transferred in a three-neck round-bottom flask. The solution was purged with nitrogen for 1 h at room temperature, at medium stirring rate and then heated to 70 °C. Potassium persulfate (KPS, Sigma-Aldrich, 46 mg, 0.17 mmol) was dissolved in 5 mL of H_2O and was added to the solution to initiate the polymerization. The reaction was maintained at 70 °C under nitrogen for 4 h. Particles were washed five times by centrifugation (19,000 rpm, 50 min, 25 °C) to eliminate the unreacted monomer and then re-suspended in 100 mL of H_2O

4.1.2. 77% NIPAm / 8% BIS / 8% DHEA/ 7% AAc

Acrylic acid was added to the particles in order to provide an available chemical moiety that would allow us to covalently bind an organic reactive dye to the nanoparticles. Poly (*N*-Isopropylacrylamide –co- Acrylic Acid), poly (NIPAm-co-AAc) particles contain an available carboxylic group that can be used in an amidation reaction to bind amino-containing dyes.

N-Isopropylacrylamide (NIPAm, Sigma-Aldrich, 697mg, 6.16 mmol) and 1,2 – Dihydroxyethylene bis-acrylamide (DHEA, Sigma-Aldrich 128mg, 0.64mmol), *N*,*N*′-methylene bisacrylamide (BIS Sigma-Aldrich, 99mg, 0.64 m mol) and acrylic acid (AAc, Sigma-Aldrich, 38 μL, 0.56m mol) were dissolved in 65 mL of H_2O, filtered using a nitrocellulose membrane disk filter (0.45 μm pore size, Millipore), and transferred in a three-neck round-bottom flask. The solution was purged with nitrogen for 1 h at room temperature, at medium stirring rate, and then heated to 70 °C. Potassium persulfate (KPS, Sigma-Aldrich, 46 mg, 0.17 mmol) was dissolved in 5 mL of H_2O and was added to the solution to initiate the polymerization. The reaction was maintained at 70 °C under nitrogen for 4 h. Particles were washed five times by centrifugation (19,000 rpm, 50 min, 25 °C) to eliminate the unreacted monomer and then resuspended in 70 mL of H_2O.

4.2 Covalent incorporation of trypan blue in poly(NIPAm-co-AAc) by zero-length amidation reaction

Trypan Blue dye, containing an amine group, was coupled by amidation reaction to the carboxylic group of acrylic acid present in the poly (NIPAm-co-AAc) particles. Briefly, 1.0g of trypan blue was dissolved in 100ml of 0.2M Na_2HPO_4 pH 9. The dye solution was centrifuged for 50 min at 19,000 rpm at 25C. Precipitates consisting of undissolved dye were discarded and supernatant was saved for the coupling reaction. Activation of the carboxylic group present in the nanoparticles was performed as follows. 10 mL of poly (NIPAm-co-AAc) particle

suspension was centrifuged (16.1 rcf, 25 C, 15 min), the supernatant was discarded, and the particle pellet was resuspended in 10 mL of 0.2 M NaH_2PO_4 pH 5. Particle suspension was transferred in a three-neck round flask, and to this were added 1 mL of 1% SDS (w/v). An activator solution was prepared as follows: 260 mg of *N*-(3 dimethylaminopropyl)-*N′*-ethyl carbodiimide hydrochloride (EDC; Fluka Analitical), and 150 mg of solid *N*-hydroxy succinimide (NHS; Sigma-Aldrich) were solubilized in 0.2 M NaH2PO4 pH 5. Nanoparticle suspension was mixed with activator solution and the reaction was held for 15 min. Particle suspension was centrifuged (19,000 rpm, 50 min, 25 °C), supernatant was discarded and particles were re-suspended in the 0.2M Na_2HPO_4 pH 9 buffer containing trypan blue. The reaction was held at room temperature at medium stirring rate overnight. In order to eliminate the unreacted dye, poly (NIPAm/ trypan blue), poly (NIPAm/TB) particles were washed five times with water by centrifugation (19,000 rpm, 50 min, 25 °C). Supernatants were discarded, and particles were re-suspended in 10 mL of water.

4.3 **Degradation of particles containing DHEA as crosslinker**.

Particles containing DHEA were subjected to oxidation by NaIO4 solution in order to degrade DHEA cross links. 5 mL of particle suspension was mixed with 5 mL of NaIO4 dissolved in 0.05M citrate buffer pH 5.0 for 10 minutes. DHEA and NaIO4 molar ratio was kept 1:1. Molar content of the different kinds of particles is reported in Table 4.1

Table 4.1: NaIO4 oxidative degradation of DHEA containing NIPAm particles was performed keeping DHEA: NaIO4 molar ratio 1:1. Molar quantities are reported.

Molar % of DHEA	DHEA (mg)	DHEA(mmole)	NaIO4(mg)	NaIO4(mmoles)
12%	169mg	0.84	179.66	0.84
8%	128mg	0.64	136.88	0.64

4.4. Nanoparticle characterization by light scattering

Average particle size was determined at each step of particle synthesis using photon correlation spectroscopy (N5 Submicron Particle Size Analyzer, Beckman Coulter). The measurement of the particles was performed using an equilibration time of 10 min and 200 s integration times. Water was used as the diluent (refractive index (RI) = 1.333, diluent viscosity = 0.890 cP), and the test angle for all light scattering experiments was 90°. Each measurement was performed in triplicate, and the average mean diameter for each was determined by converting the observed values to particle sizes via the Stokes-Einstein relationship;

$$D = \frac{\bar{R}T}{N_A} \frac{1}{6\pi\eta a}$$

4.5. Protein sieving and protein preservation by dually crosslinked, trypan blue functionalized particles

Protein binding and sieving properties of the NIPAm/TB particles were evaluated by incubating particles with a solution containing proteins with different molecular weight. The protein solution contained the following proteins: aprotinin (MW 6500 Da, Sigma-Aldrich), lysozyme (MW 14,400 Da, Sigma-Aldrich), carbonic anhydrase (MW 31,000 Da, Sigma-Aldrich) and BSA (MW 66,000 Da, Fisher Scientific) dissolved in Dulbecco's Phosphate-Buffered Saline (PBS, Gibco) at a concentration of 0.08 mg/mL). Particles were allowed to incubate with protein solution for 30 minutes at room temperature. After the 30 min incubation period, Particle suspensions were centrifuged at 16,100 rcf for 7 min at 25C in order to separate the particles from the aqueous medium. Supernatant was discarded and pellet was washed with water. More in detail, pellet re-suspended in 500 ul of water and centrifuged at 16,100 rcf, for 7 minutes at 25C. Particle pellet was re-suspended in 10 ul of 2X SDS running buffer (Invitrogen) and placed in heating block for 5 minutes at 100C. Samples including initial protein solution, particle supernatant and particle pellet were subjected to SDS PAGE analysis (20% Tris-glycine, Invitrogen). Silver staining was used to stain the gels.

Image analysis of each gel was performed by capturing the image of each gel with a PC scanner (HP ScanJet 5400c) and saving it as graphic files in JPEG format.

In order to assess if proteins captured by the particles were protected from oxidative degradation, particles incubated with the protein solution described above were treated with 20 ul of NaIO4 dissolved in 0.05M citrate buffer pH 5 for 10 min. DHEA:NaIO4 molar ratio was kept 1:1 (0.64 m moles). After 10 minutes, NaIO4 treated hydrogel particles were centrifuged at 16,100 rcf for 7 min to separate the particles from the NaIO4 buffer and re-suspended in 25uL PBS. Particle pellet was washed and analyzed by SDS PAGE as described above.

4.6. IL-6 Antibody validation by western blot

In order to ensure valid ELISA results, validation of a commercially available IL-6 monoclonal antibody (Abcam) was performed with Western Blot analysis. Different concentration of IL-6 protein (Biolegend) were spiked in human urine in order to determine if the IL6 antibody presented cross-reactivity with other proteins present in urine. Protein solutions were separated by 1-D gel electrophoresis in 4-20% Tris-Glycine gel. Proteins were then transferred onto Immobilion PVDF membrane. The membrane was then incubated with PBS supplemented with 10% I-Block and 0.1% Tween 20 for 1 hour at room temperature, and then with horseradish peroxidase conjugated IL-6 antibody (Abcam) raised against IL6 overnight at 4 °C under continuous agitation. After washes

with PBS supplemented with 0.2% I-Block (w/v) and 0.1% Tween 20, immunoreactivity was revealed by using a the enhanced chemiluminescence system (Pierce). The protein blot was imaged using Kodak 4000MM.

4.7. ELISA procedure using degradable, trypan blue functionalized particles.

50uL (NIPAm/TB) particles were incubated in triplicate for 30 min with 1 ml of IL-6 protein spiked in human urine at different concentrations ; 0.6ng/ml, 1.25ng/ml, 2.5ng/ml, 5ng/ml, 10ng/ml, 20ng/ml and 50ng/ml. After incubation, particles were washed with PBS as follows: particles were centrifuged (16,100 rcf, 7 minutes, 25C), and supernatant was removed; particles were re-suspended with 50uL PBS, centrifuged (16,100 rcf, 7 minutes, 25C), and supernatant was discarded.50ng/mL IL-6 incubated NIPAm/TB Particles were treated with 50uL of 0.64 molarNaIO4 in 0.05M citrate buffer pH 5 for 5 min Particles were washed with PBS as described above. Then, particles were incubated with I block (0.2% I-Block (w/v) and 0.1% Tween 20) for 30min and washed with 50uL of PBS as described above. Particle pellets were re-suspended in 50uL of PBS. Particle suspensions were incubated with horseradish peroxidase conjugated antibody raised against IL6 for 2 hours at room temperature under continuous agitation. In order to separate poly (NIPAm/TB) particles from the excess antibody in solution, magnetic separation was pursued. In detail, 30uL magnetic nanoparticles (Fe3O4 nanoparticles, Ø 100nm, coated with oleic acid, Chemicell) were added to poly(NIPAm/TB) particles incubated with the IL-6 antibody solution. Suspensions of these particles were transferred to an ELISA 96 well plate and let incubate for 10 minutes.

Magnetic Fe3O4 nanoparticles are non covalently associated to poly (NIPAm/TB) particles and permit separation under magnetic field. After 10 minutes incubation, the 96 well plate was placed on neodymium magnets. In few seconds poly (NIPAm/TB) particles and magnetic nanoparticles adhered to the bottom of the wells. Supernatants were removed. Particles were re-suspended with100 ul PBS, separated on the neodymium magnet and re-suspended in 100ul of PBS. 100 µl of TMB Reagent (3,3′,5,5′-Tetramethylbenzidine, Thermo scientific) were added to each well and let react for 10 minutes at room temperature under gentle mixing.50 µl of Stop solution(0.1M HCl) were added to the wells. Stop buffer was allowed to react to 10~15 minutes. Absorbance of each well was read at 450 nm using Instrument elisa plate reader.

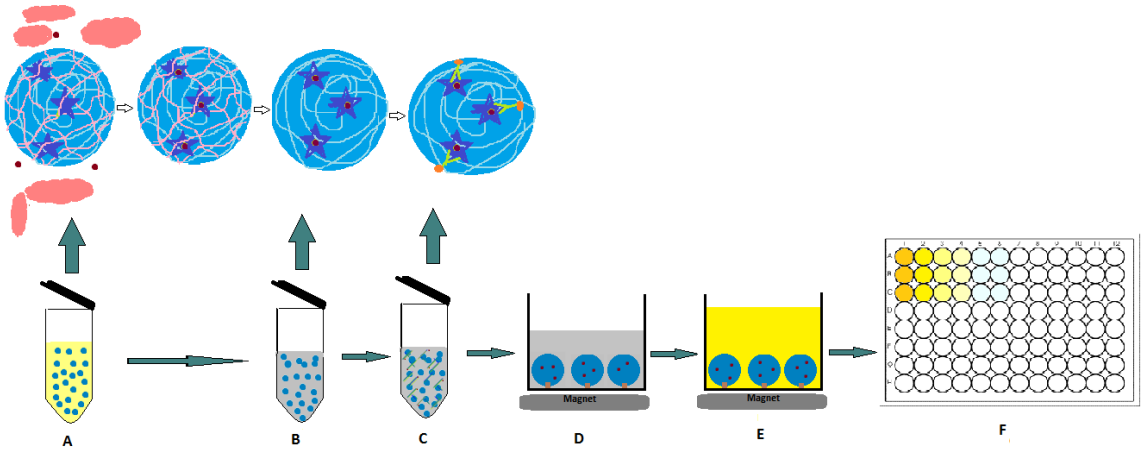


Fig. 4.1: Scheme of IL6 ELISA based on partially degradable poly (NIPAm/TB) particles. Poly (NIPAm/TB) particle are incubated with human urine spiked with recombinant IL6. In few minutes poly (NIPAm/TB) particles capture all the solution phase IL6 DHEA crosslinkers are degraded by NaIO4. This step causes the pore size of poly (NIPAm/TB) particles to increase and allow the antibody to penetrate the interior space. Particles are mixed to anti-IL6 antibody. Poly (NIPAm/TB) particles are transferred to an ELISA plate and magnetically separated from excess, unbound antibody. TMB reagent is added to the poly (NIPAm/TB) particles and absorbance is read.

4.8. LC-MS MS Analysis of poly (NIPAm/TB) particle washing in order to exclude IL-6 release from the capturing particles

As described above, poly (NIPAm/TB) particles incubated with IL6 solutions are washed with PBS in order to remove analytes that non-specifically interact with the particles and that precipitate under high speed centrifugation. In order to assess whether these washing steps cause any loss of IL6 with subsequent loss of signal in the ELISA, assessment of supernatants derived from particle washing were performed with Mass Spectrometry. 50ul of poly(NIPAm/TB) particles were incubated with 1 ml of IL6 spiked in urine at the following concentrations: 1ng/ml, 6ng/ml and 9ng/ml. Particles were separated by centrifugation (16,100 rcf, 7 minutes, 25C) and re-suspended in 50 ul PBS. . Particles were centrifuged (16,100 rcf, 7 minutes, 25C) and supernatant was saved for mass spectrometry analysis. A positive control was also prepared containing IL6 at 0.05 ug/mL in PBS. All samples were denatured with 8 M urea, reduced by 10 mM dithiothreitol (DTT), alkylated by 50 mM iodoacetamide, and digested by trypsin (1ug trypsin Promega/20ul of sample containg 1ng, 6ng and 9ng IL-6). Tryptic peptides were further purified by Zip-Tip (Millipore) and analyzed by reversed-phase liquid chromatography nanospray tandem mass spectrometry (LC–MS/MS) using an LTQ-Orbitrap mass spectrometer (ThermoFisher).

After sample injection by autosampler, the C18 column (0.2 mm × 50 mm, Michrom Bioresources, Inc.) was washed for 2 minutes with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 50% B in 90 minutes at 1.5 uL/minute, then with 100% B for an additional 5 minutes. The LTQ-Orbitrap mass spectrometer was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The Dynamic Exclusion Time was 30 s, and the Dynamic Exclusion Size was 200. The "FT master scan preview mode", "Charge state screening", "Monoisotopic precursor selection", and "Charge state rejection" were enabled so that only the 1+, 2+, and 3+ ions were selected and fragmented by CID.

Mass spectrometry data analysis

Tandem mass spectra collected by Xcalibur (version 2.0.2, Thermo Scientific) were searched against the NCBI Bovine and Human protein database (released in September 2009 with 37391 entries) using SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, static cysteine alkylation by iodoacetamide, and variable methionine oxidation. Mass tolerance for precursor ions was 5 ppm and mass tolerance for fragment ions was 0.25 Da. The SEQUEST search results of proteomics data were filtered by the criteria "Xcorr versus charge 1.9, 2.2, 3.0 for 1+,

2+, 3+ ions; ΔCn > 0.1; ranked top #1; probability of randomized identification of peptide < 0.01".The SEQUEST search results were exported to Excel files.

CHAPTER 5

RESULTS AND DISCUSSION

5.1. Nanoparticle Characterization by light scattering

In order to assess the hydrodynamic diameter of NIPAm based particles and to verify if oxidative degradation of degradable DHEA crosslinkers had an effect on particle size, light scattering analysis was performed. Particles obtained with the following molar ratio of reactants were studied: 1) 88% NIPAm/ 12% DHEA particles, and 2) 77% NIPAm/ 8% BIS / 8% DHEA / 7%AAc particles functionalized with trypan blue. Particle size was assessed at 25C in water and particles exhibited homogeneous size distribution. Results reported in Figure 5.1 show that NaIO4 treatment causes a percentage size increase of 8% and 35% in 88% NIPAm/ 12% DHEA particles and 77% NIPAm/ 8% BIS / 8% DHEA / 7%AAc particles functionalized with trypan blue, respectively. It is hypothesized that the breakage of DHEA crosslinks results in a particle architecture that is more open and therefore allowing a higher number of water molecules to penetrate in the inner space, this resulting in an increase hydrodynamic diameter.

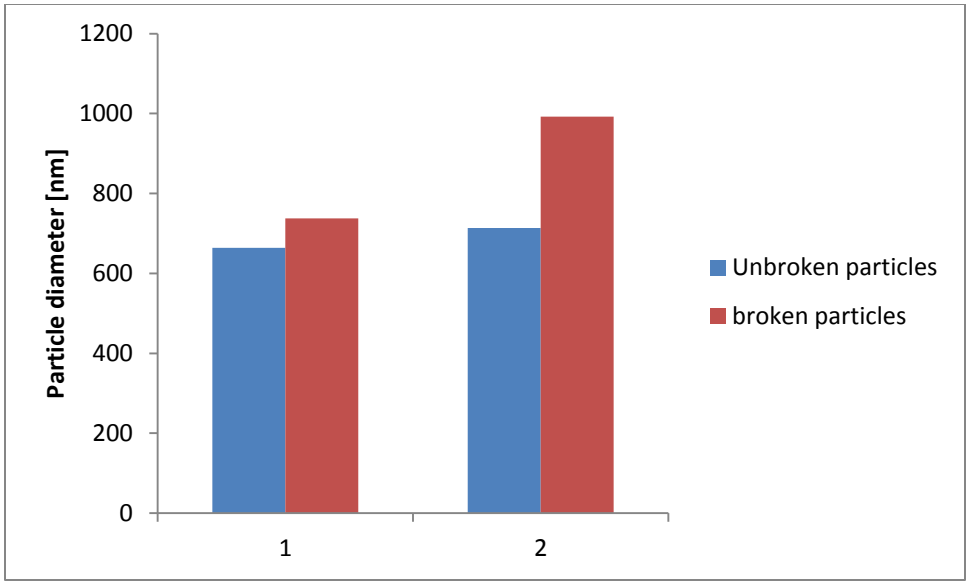


Fig. 5.1: NaOI4 treatment changes the hydrodynamic diameter of NIPAm particles containing DHEA degradable crosslinker. 1. 88% NIPAm/12% DHEA particles 2. 77% NIPAm/ 8% BIS / 8% DHEA / 7%AAc functionalized with trypan blue

5.2 Protein sieving and protein preservation by dually crosslinked, trypan blue functionalized particles

To assess the molecular sieving properties of poly (NIPAm/TB) particles, particles were incubated with a solution containing the following proteins at a concentration of 0.08 mg/mL: aprotinin (MW 6500 Da), lysozyme (MW 14,400 Da), carbonic anhydrase (MW 31,000 Da) and BSA (MW 66,000 Da). The uptake of proteins by the particles was evaluated by SDS PAGE analysis. According to SDS PAGE results presented in Fig 5.2, dually crosslinked poly (NIPAm/TB) particles captured and concentrated low molecular weight proteins whereas proteins with high molecular weight remained excluded from the particles (Figure 5. 2, lanes PS and P). NaIO4 treated poly (NIPAm/TB) particles have a

higher capturing capacity with respect to untreated poly (NIPAm/TB) particles (Figure 5.2, lanes BPS and BP).

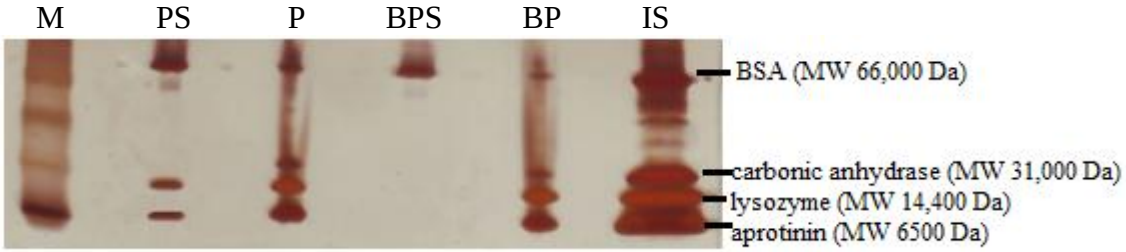


Fig 5.2. SDS PAGE analysis shows that NaIO4 treated poly(NIPAm/TB) particles have a higher capturing capacity with respect to untreated poly(NIPAm/TB) particles; M molecular weight marker, PS supernatant for untreated particles, P untreated particles. BPS NaIO4 treated particle supernatant, BP NaIO4 treated particles and IS initial solution of proteins different molecular weights.

In order to study the effects of NaIO4 treatment (DHEA degradation) on the protein captured by poly (NIPAm/TB) particles, the following experiment was performed:poly(NIPAm/TB) particles were incubated with a solution containing: aprotinin (MW 6500 Da), lysozyme (MW 14,400 Da), carbonic anhydrase (MW 31,000 Da) and BSA (MW 66,000 Da) each at a concentration of 0.08 mg/mL. The protein/particle suspension was subjected to NaIO4 treatment for 10 minutes. Control poly (NIPAm/TB) particles/protein suspensions were left untreated. Particles were washed with PBS and samples were subjected to SDS PAGE analysis. Results presented in Fig 5.3 show that both particles sequestered small size protein equally. And NaIO4 treatment for DHEA degradation has no impact on captured protein integrity.

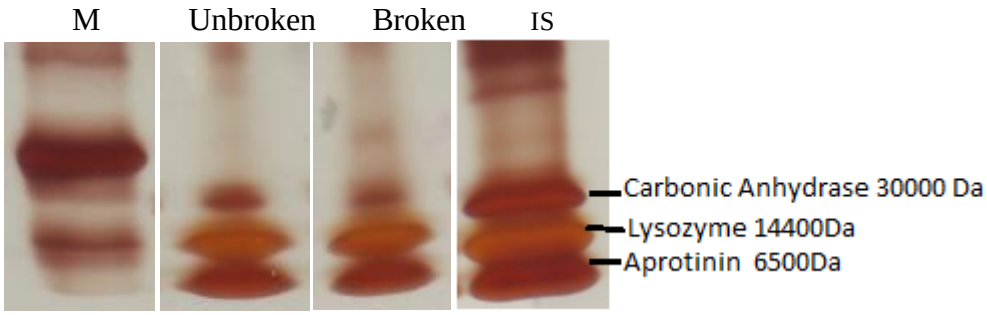


Fig 5. 3. SDS PAGE analysis shows that protein captured by poly(NIPAm/TB) particles are protected from oxidative degradation; M molecular weight marker, Untreated: untreated poly(NIPAm/TB) particle content, Treated: NaIO4 treated poly(NIPAm/TB) particle content, IS initial solution of protein with different molecular weights.

5.3. IL-6 Antibody validation by western blot

In order to obtain reliable ELISA results, it is common practice to validate the antibodies used in order to exclude the possibility of cross-reaction with non specific protein antigens. IL-6 antibody was validated by western blot by using IL6 recombinant protein spiked at different concentration in human urine. This model solution allowed us to determine the specificity of the chosen, commercial antibody in presence of excess competing proteins in human urine, which is the biofluid we will be using for our ELISA test. Results presented in Fig 5.4 indicate that IL-6 antibody has a very good specificity for IL6 in presence of excess competing urinary proteins; bands are visible for IL-6 concentrations ranging from 200ng/ml to 25ng/ml.

These findings indicate that IL-6 antibody has high affinity towards IL-6 protein used in various concentrations.

M 200ng 100ng 50ng 25ng 12ng 6ng

Fig 5.4 Western blot analysis shows that the IL-6 antibody we chose for this study has a very good specificity for the target analyte. Dose response curve shows linearity of response in a 200 to 25 ng range

5.4. ELISA

In our novel ELISA setting, poly (NIPAm/TB) particles were incubated with human urine spiked with different concentrations of IL-6 recombinant protein. Particles were harvested by centrifugation and washed in order to eliminate non specific binding. Particles were treated with NaIO4 in order to degrade DHEA crosslinkers and increase the pore size in order to allow the antibody to access the inner space of particles and interact with the captured antigen. Treated particles were treated with blocking solution in order to block all the non specific binding sites. Particles were incubated with the HRP labeled IL6 monoclonal antibody and treated chromogenic substrate. The colorimetric signal was detected by using optical density. A standard curve for this IL6 ELISA assay based on partially degradable poly (NIPAm/TB) particles was generated in order to assess the quality of the procedure. A linear trend was obtained in the range from 20000-600 pg; the limit of detection for current setting is therefore 600 pg. (Figure 5.6). Blocking of binding sites improves the background signal as demonstrated by the comparison of NaIO4 treated particles incubated with blocking solution with NaIO4 treated particles without blocking solution. This result confirms the need of a blocking step in our ELISA protocol. (Table 5.1).

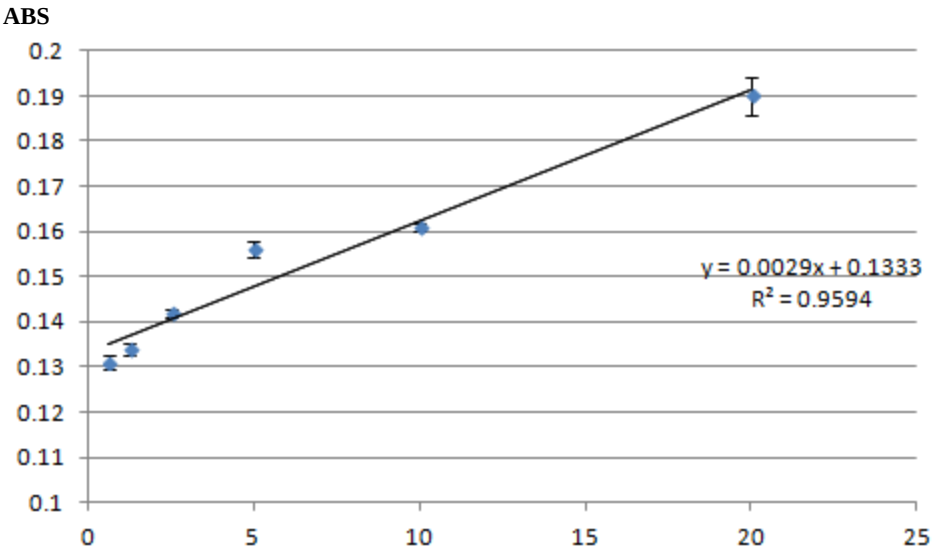


Fig. 5.5. Calibration curve of IL-6 ELISA based on partially degradable poly(NIPAm/TB) particles shows linearity over a 3 orders of magnitude and shows %coefficient of variations constantly below 5%. Average +/- standard deviation over three experimental replicates.

Table 5.1. Blocking of binding sites improves the background signal as demonstrated by the comparison of NaIO4 treated particles incubated with blocking solution with NaIO4 treated particles without blocking solution.

No.	NaIO4 treated Particle with I-Block	NaIO4 treated Particle without I-Block	I-Block
1.	0.063	0.109	0.033
2.	0.059	0.114	0.039
3.	0.060	0.112	0.036
Average	0.06	0.111	0.033
S.D	0.002	0.0025	0.003

This result confirms the need of a blocking step in our ELISA protocol.

5.5. Degradation of DHEA crosslinker causes particle pore size to increase and allows antibody to access IL6 antigen in the inner space of particles.

In order to test if the NaIO4 treatment is effective in increasing the pore size and allowing the antibody to access the inner space of the particles, the following experiment was performed. Poly(NIPAm/TB) particles were incubated with 1ml of human urine spiked with 50ng of IL-6 recombinant protein. Results shown in Fig 5.5 demonstrate that the signal obtained from treated particles is two fold with respect to untreated particles and that untreated particles yield a signal that is very close to background signal (absence of IL6 recombinant proteins). This confirms that the NaIO4 treatment allows the monoclonal antibody to interact with IL6 antigen at a higher extent.

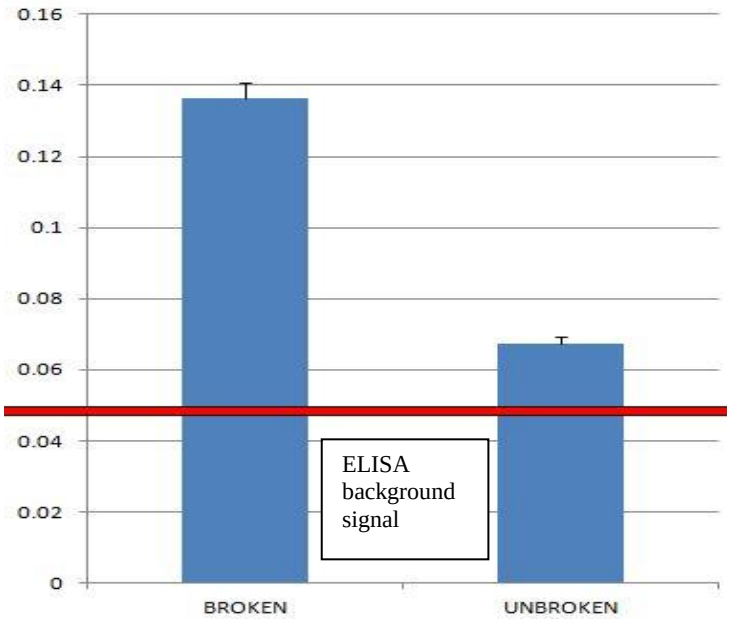


Fig 5.6: ELISA analysis shows that NaIO4 treatment of poly (NIPAm/TB) particles allows the IL6 antibody to access and react with IL6 antigen captured in the inner space of the particles.

In fact, absorbance signal obtained from NaIO4 treated poly (NIPAm/TB) particles is 200% of the signal obtained from untreated poly(NIPAm/TB) particles. Moreover, signal obtained from untreated poly (NIPAm/TB) particles is at the level of ELISA background signal, indicating that there is negligible antibody/antigen interaction when the particles are untreated and the tight pore size impedes antibody access to the inner space of the particles.

5.6 . LC-MS MS Analysis of poly(NIPAm/TB) particle washing solution in order to exclude IL-6 release from the capturing particles:

Poly (NIPAm/TB) particles were incubated in three different IL-6 samples with different concentrations: 1ng/ml, 6ng/ml and 9ng/ml. After incubation, particles were washed and incubated with a blocking solution constituted by 0.2% I-Block (w/v) and 0.1% Tween 20 . Particles were washed with 100 uL of PBS as described in the Materials and Methods. Particles were pelleted and the supernatant (washing solution) was subjected to mass spectrometry analysis in order to detect if traces of IL6 leaking out of the particles were detectable. LC MS/MS analysis of the washing solution did not reveal the presence of any peptide belonging to IL-6 (Table 5.3-5.10). This finding signifies robustness of the protocol and indicates binding affinity of the trypan blue towards IL-6. Example chromatograms and MS/MS spectra are reported in Fig. 5.7 in order to document the quality of the mass spectrometry runs.

Table 5.2. Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.05 ng/ul of IL6 Positive control1, (human data base)

Reference	P (pro)	MW	Accession	Spectra count
peptidoglycan recognition protein 1 [Homo sapiens]	5.63E-09	21716.7	4827036	1
salivary amylase alpha 1C precursor [Homo sapiens]	1.53E-08	57731.0	56549664	1
insulin-like growth factor binding protein 7 [Homo sapiens]	2.58E-06	29111.5	4504619	1
SH3 domain binding glutamic acid-rich protein like 3 [Homo sapiens]	2.78E-06	10431.3	13775198	1
sulfatase 2 isoform b precursor [Homo sapiens]	4.33E-06	98120.0	38327658	1
regenerating islet-derived 1 alpha precursor [Homo sapiens]	8.80E-06	18718.8	29725633	1
PREDICTED: similar to immunoglobulin lambda-like polypeptide 1 [Homo sapiens]	3.01E-05	53218.3	239752604	1
leucine-rich alpha-2-glycoprotein 1 [Homo sapiens]	3.10E-05	38154.1	16418467	1
ubiquitin and ribosomal	4.47E-05	14719.0	77539055	1

protein L40 precursor [Homo sapiens]				
pancreatic ribonuclease precursor [Homo sapiens]	5.46E-05	17632.7	38201682	1
keratin 6B [Homo sapiens]	1.74E-04	60030.3	119703753	1
alpha 1B-glycoprotein precursor [Homo sapiens]	2.62E-04	54219.7	21071030	1
tumor endothelial marker 1 precursor [Homo sapiens]	3.36E-04	80807.5	9966885	1
clusterin isoform 1 [Homo sapiens]	5.37E-04	57795.7	42716297	1
mannan-binding lectin serine protease 2 isoform 2 precursor [Homo sapiens]	6.19E-04	20615.9	21264361	1
orosomucoid 2 [Homo sapiens]	6.60E-04	23587.6	4505529	1

Table 5.3 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.05 ng/ul of IL6 , (human data base)

Reference	P (pro)	MW	Accession	Spectra count
keratin 1 [Homo sapiens]	2.09E-07	65998.9	119395750	6
fatty acid binding protein 3 [Homo sapiens]	7.70E-11	14848.7	4758328	3
keratin 2 [Homo sapiens]	3.84E-08	65393.2	47132620	2
keratin 9 [Homo sapiens]	3.16E-07	62026.7	55956899	2
lipoprotein lipase precursor [Homo sapiens]	3.70E-11	53128.9	4557727	1

Table 5.4. Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.3 ng/ul of IL6 , (human data base)

intermediate filament tail domain containing 1 isoform 1 [Homo sapiens]	4.97E-05	45862.2	224593273	3
fatty acid binding protein 3 [Homo sapiens]	1.28E-08	14848.7	4758328	2
albumin preproprotein [Homo sapiens]	1.55E-05	69321.6	4502027	2
Trypsin_porcine	5.31E-13	53128.9	4557727	1
lipoprotein lipase precursor [Homo sapiens]	5.31E-13	53128.9	4557727	1
lactoperoxidase isoform 1 preproprotein [Homo sapiens]	5.95E-07	80237	40549418	1

Table 5.5 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.45 ng/ul of IL6, (human data base)

Reference	P (pro)	MW	Accession	Spectral Count
albumin preproprotein [Homo sapiens]	7.59E-10	69321.6	4502027	1
milk fat globule-EGF factor 8 protein isoform a [Homo sapiens]	2.65E-08	43077.5	167830475	1
xanthine dehydrogenase [Homo sapiens]	4.80E-06	146329.8	91823271	1
intermediate filament tail domain containing 1 isoform 1 [Homo sapiens]	1.19E-04	45862.2	224593273	1

Table 5.6 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.05 ng/ul of IL6 Positive control, (bovine data base)

Reference	P(pro)	Sf	Score	MW	Accession	Peptide hit
albumin [Bos taurus]	1.08E-11	1.91	20.22	69278.6	30794280	3 (3 0 0 0 0)
casein alpha s1 [Bos Taurus]	4.14E-09	1.88	20.16	24513.4		2 (2 0 0 0 0)
D Chain D, Crystal Structure Of Deoxy Human Hemoglobin	1.10E-05	0.94	10.14	16342.1	37926924	1 (1 0 0 0 0)
A Chain A, Bovine Prothrombin Fragment 1	1.39E-05	1.84	20.19	8559.6	38373984	2 (2 0 0 0 0)
JS0638 osteopontin precursor - bovine	2.67E-05	0.51	8.7	15043.9	89697	1 (0 1 0 0 0)

Table 5.7 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.1 ng/ul of IL6 , (bovine data base)

reference	P(pro)	Sf	Score	MW	Accession	Peptide(Hit)
casein alpha-S2 [Bos Taurus]	1.00E-30	11.12	120.31	26002.3		30(300000)
A59068 beta-casein variant CnH - bovine	1.00E-30	6.74	70.31	23515.2	7441526	25(250000)
casein alpha s1 [Bos Taurus]	2.22E-16	11.41	120.32	24513.4		39(390000)
B Chain B, Bovine Beta-Lactoglobulin, Lattice X	7.22E-15	7.69	80.26	18297.5	2194890	16(160000)
S65138 glycoprotein antigen MGP57/53	2.30E-13	5.63	60.21	44734.9	2136760	16(160000)
A27053 lipoprotein lipase (EC 3.1.1.34) - bovine	3.11E-13	0.97	10.24	50519.4	1071864	1(10000)

Table 5.8 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.3ng/ul of IL6 (Bovine data base)

REFERENCE	P(Pro)	Sf	Score	MW	Accession	Peptide (HIT)
casein alpha s1 [Bos Taurus]	1.00E-30	10.50	110.31	24513.4		34(340000)
A59068 beta-casein variant CnH - bovine	1.00E-30	5.84	60.29	23515.2	7441526	21(210000)
B Chain B, Bovine Beta-Lactoglobulin	5.55E-16	5.78	60.30	18297.5	2194890	10(100000)
casein alpha-S2 [Bos Taurus]	9.99E-16	12.53	140.32	26002.3		23(230000)
A27053 lipoprotein lipase (EC 3.1.1.34) - bovine	2.14E-13	1.95	20.26	50519.4	1071864	2(20000)
F Chain F, Crystal Structure Of Bovine Alpha-Lactalbumin	2.17E-12	2.98	30.23	14176.8	12084477	5(50000)
S65138 glycoprotein antigen MGP57/53, mammary gland - bovine	5.37E-12	8.46	90.25	69278.6	30794280	12(120000)

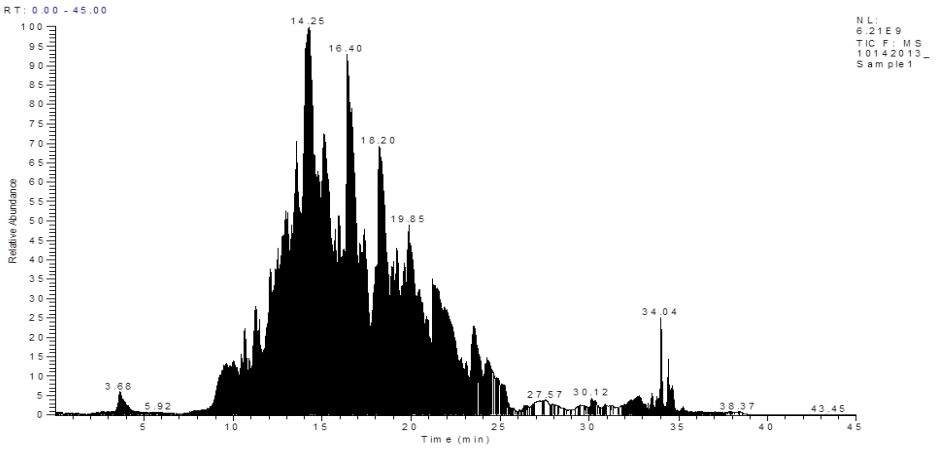
A Chain A, Nmr Study Of Bovine Heart Fatty Acid Binding Protein	2.10E-11	7.19	80.23	44734.9	2136760	10(100000)

Table 5.9 Proteins found in the washing solution of poly(NIPAm/TB) particle incubated with a solution containing 0.45 ng/ul of IL6, (Bovine data base)

Reference	P(pro)	Sf	Score	MW	Accession	Peptide (hit)
A59068 beta-casein variant CnH - bovine	1.00E-30	3.90	40.31	23515.2	7441526	10(100000)
casein alpha-S2 [Bos Taurus]	1.11E-16	8.91	100.30	26002.3		14(140000)
casein alpha s1 [Bos Taurus]	2.22E-15	8.67	90.34	24513.4		26(260000)
B Chain B, Bovine Beta-Lactoglobulin, Lattice X	9.46E-13	5.76	60.27	18297.5	2194089	8(80000)
albumin [Bos taurus]	8.97E-12	6.67	70.25	69278.6	30794280	7(70000)
C Chain C, Crystal Structure Of Xanthine Oxidase From Bovine Milk	2.07E-11	1.95	10.14	83808.2	10835431	2(20000)
S65138 glycoprotein antigen MGP57/53, mammary gland - bovine	6.69E-11	6.64	60.19	44734.9	2136760	8(80000)

A)Sample: 1ngIL-6/mL

Casein alpha-S2 [Bos Taurus]



Reference	P (pro)	Sf	Score	MW	Peptide(Hits)
Casein alpha-S2 [Bos Taurus]	1.0E-030	11.12	120.3	26002	30(30000)

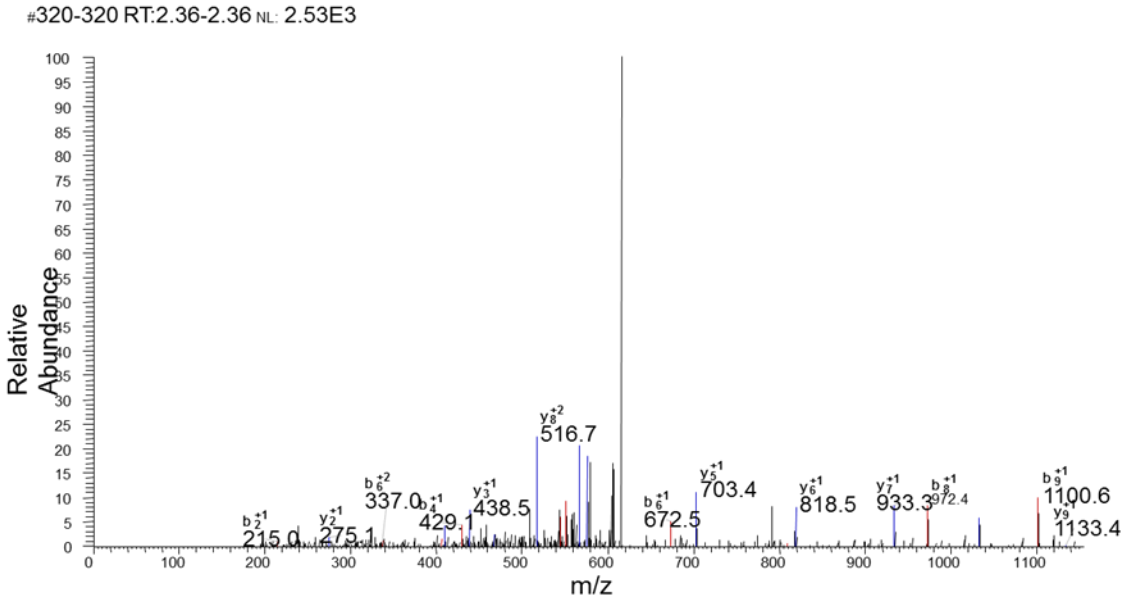
Scan(s)	Peptide	MH+	DeltaM	z	Type	P (pep)	Sf	XC
320	K. ITVDDKHYGKA	1246.263	-0.00025	2	CID	32E-008	0.88	2.90

Sp	RSp	dCn	Ion
421.0	1	0.550	12/18

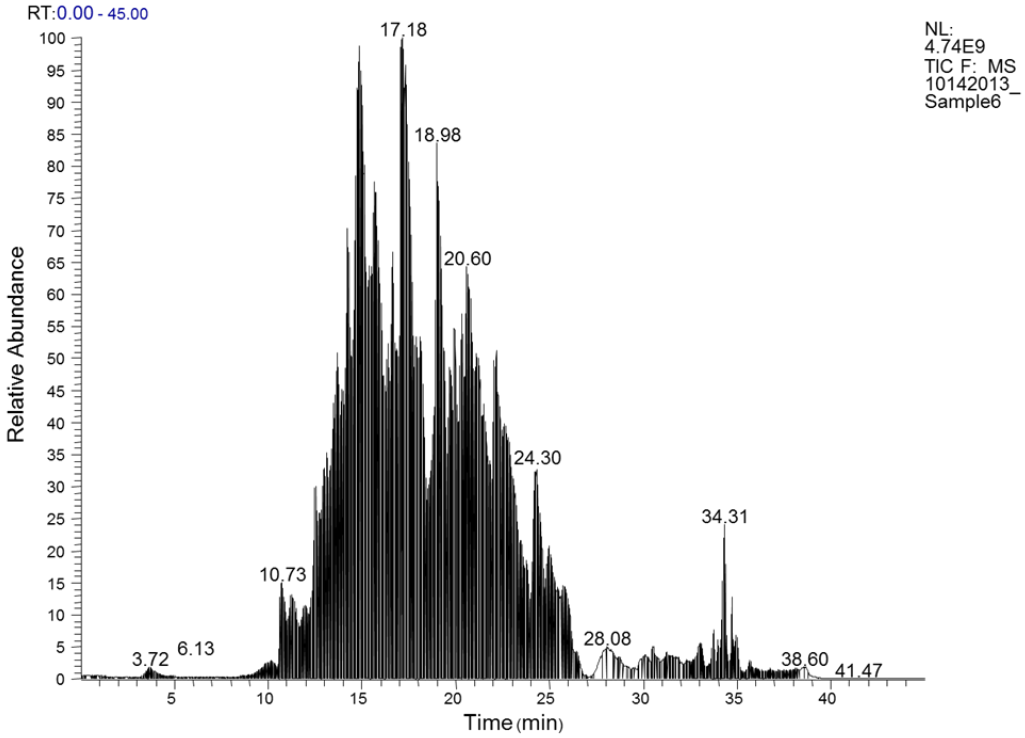
- **Casein alpha-S2 [Bos Taurus]**

Scans	peptide
320	K. ITVDDKHYGKA

AA	b	y
I	114.09134	
T	215.13902	1133.55856
V	314.20743	1032.51088
D	429.23438	933.44247
D	544.26132	818.41553
K	672.35628	703.38859
H	809.41519	575.29362
Y	972.47852	438.23471
Q	1100.5371	275.17138
K		147.11280



B) Sample: 6ng IL-6/ml
Casein alpha-S2 [Bos Taurus]



Reference	P (pro)	Sf	Score	MW	Peptide (Hits)
Casein alpha-S2 [Bos Taurus]	1.0E-015	12.53	140.3	26002	30(30000)

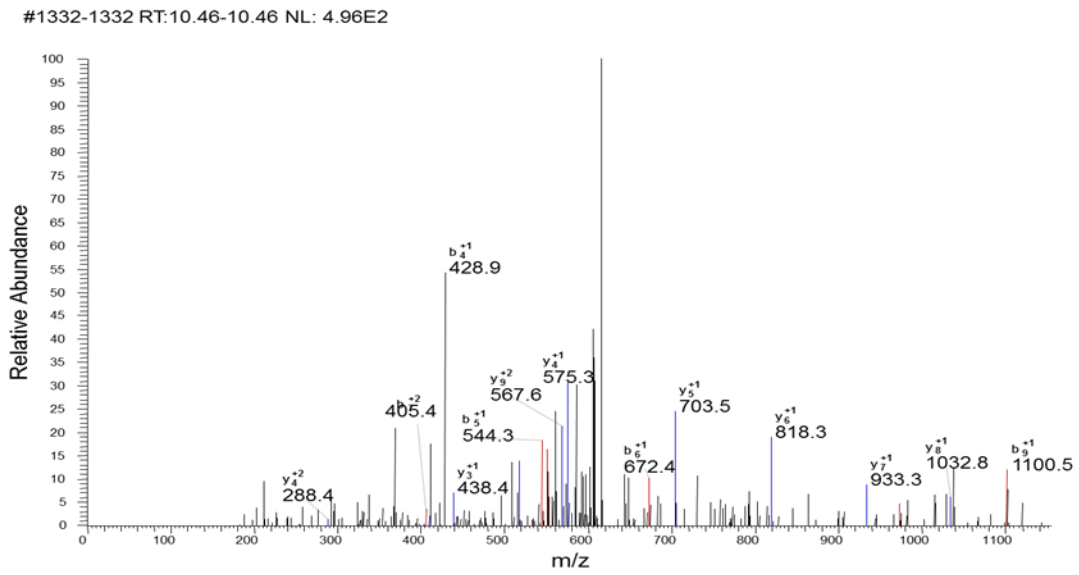
Scan(s)	Peptide	MH+	DeltaM	z	Type	P (pep)	Sf	XC
132	K. ITVDDKHYGKA	1246.263	-0.00195	2	CID	7.2E-003	0.81	2.293

dCn	Sp	RSp	Ions
0.570	427.8	1	11/18

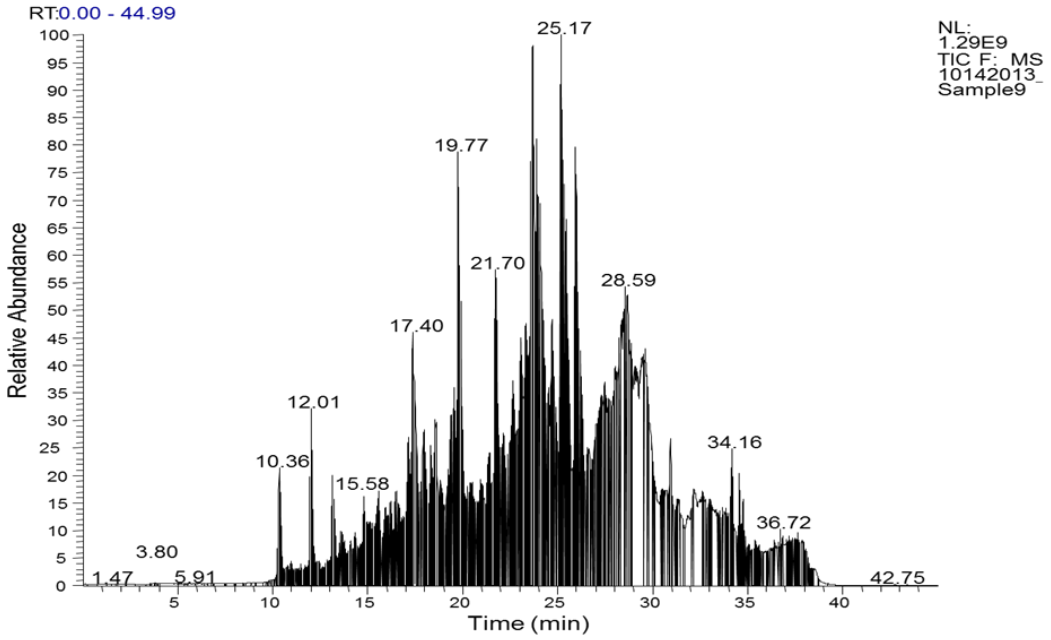
- **Casein alpha-S2 [Bos Taurus]**

Scans	peptide
132	K. ITVDDKHYGKA

AA	b	y
I	114.09134	
T	215.13902	1133.55856
V	314.20743	1032.51088
D	429.23438	933.44247
D	544.26132	818.41553
K	672.35628	8703.3859
H	809.41519	575.29362
Y	972.47852	438.23471
Q	1100.53710	275.17138
K		147.11280



C) Sample: 9ng IL-6/ml
Casein alpha-S2 [Bos Taurus



Reference	P (pro)	Sf	Score	MW	Peptide (Hits)
Casein alpha-S2 [Bos Taurus]	1.1E-016	3.9.	100.3	26002.3	30(30000)

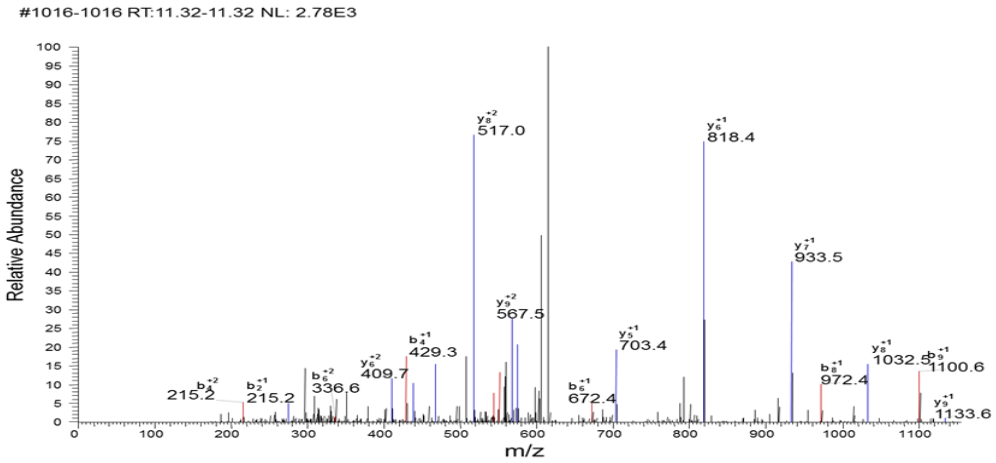
Scan(s)	Peptide	MH+	DeltaM	z	Type	P (pep)	Sf	XC
1016	K. ITVDDKHYGKA	1246.64	-0.00208	2	CID	2.4E-009	0.95	2.293

dCn	Sp	RSp	Ions
0.570	528.5	1	14/18

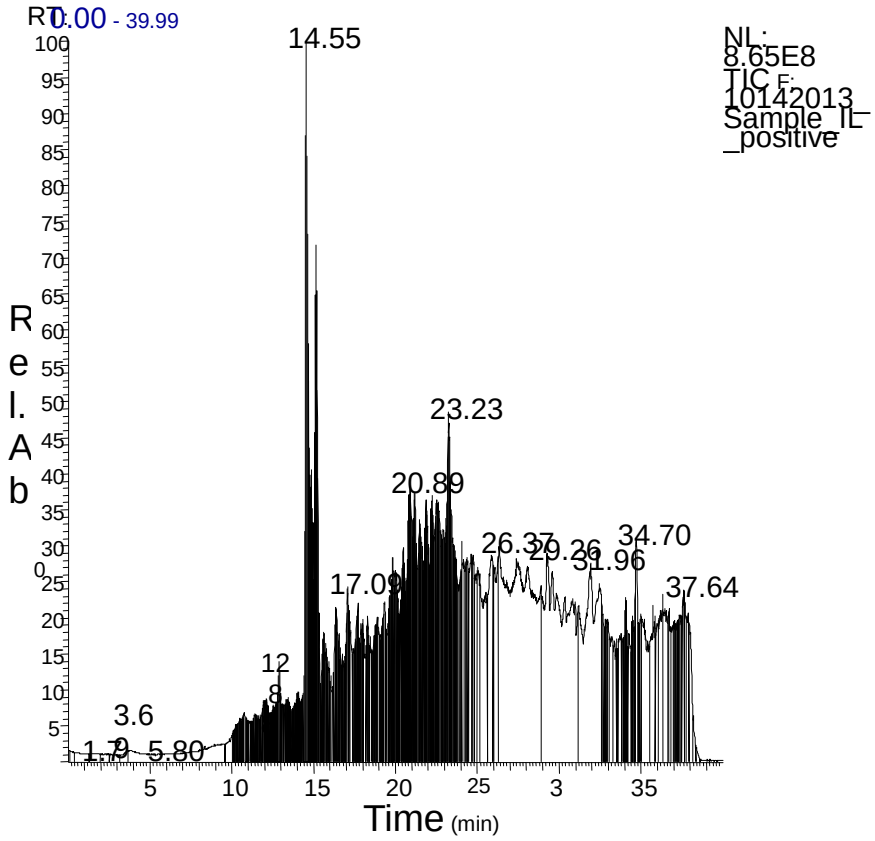
- **Casein alpha-S2 [Bos Taurus]**

Scans peptide
1016 K. ITVDDKHYGKA

AA	b	y
I	114.09134	
T	215.13902	1133.55856
V	314.20743	103251088
D	429.23438	933.44247
D	544.26132	818.41553
K	672.35628	703.38859
H	809.41519	575.29362
Y	972.47852	438.23471
Q	1100.53710	275.17138
K		147.11280



(D) Positive control(1 ng IL-6/ml)
Alpha 2-globulin [Homo sapiens]



Reference	P (pro)	Sf	Score	MW	Accession
Alpha 2-globulin [Homo sapiens]	1.5E.01	1.79	20.3	15116.9	1335076

Peptide (Hits)
3(2100)

Scan(s)	Peptide	MH+	DeltaM	z	Type	P (pep)	Sf
1497	K.VGAHAGAEALER	1529.7430	-0.000395	3	CID	2.4E006	0.49

XC	dCn	Sp	RSp	Ions
5.185	0.761	1707.6	2	28/66

- **Alpha 2-globulin [Homo sapiens]**

Scans	Peptide
1497	VGAHAGEYGAEALER

AA	b	y
V	100.07569	
G	157.09715	1430.66558
A	228.13427	1373.64442
H	365.19318	1302.60730
A	436.23029	116.54839
G	493.25176	1094.51128
E	622.29435	1037.48882
Y	785.35768	908.44722
G	842.37914	745.83369
A	913.41626	688.36243
E	1042.45885	617.325532
A	1113.49596	408.28272
L	1226.58003	417.24561
E	1355.62262	304.16155
R		175.11895

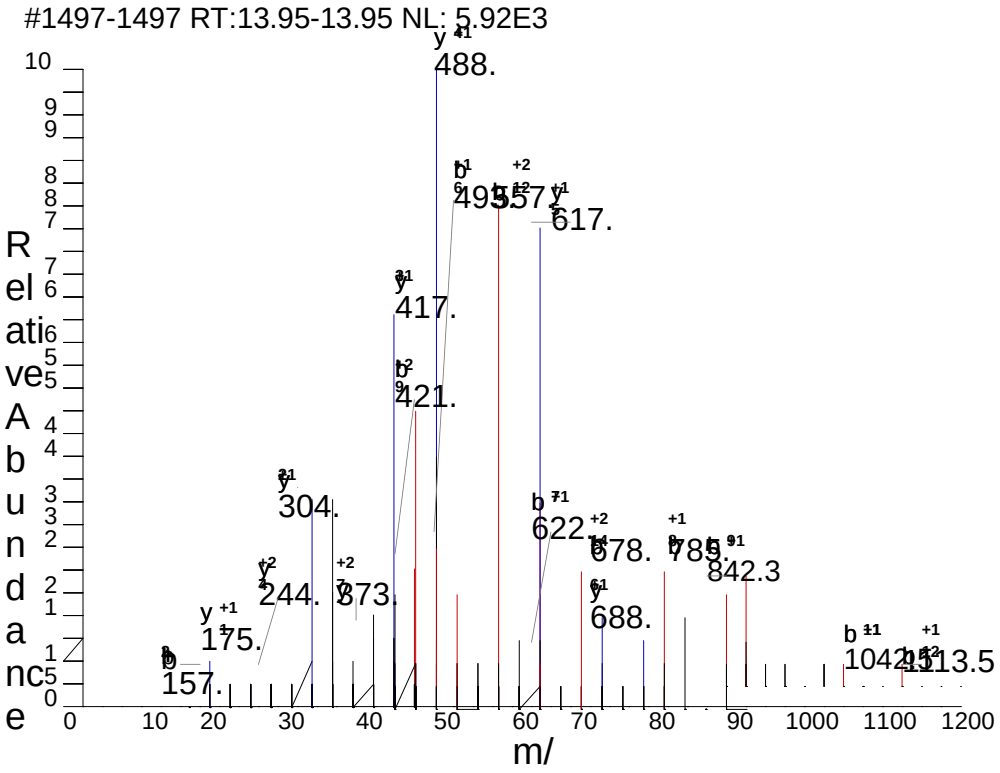


Figure5.7. Example chromatograms and MS/MS spectra from the mass spectrometry analysis of poly(NIPAm/TB) particle washing solutions. A)Sample: 1ngIL-6/mL,B) Sample: 6ng IL-6/ml, C) Sample: 9ng IL-6/ml(D) Positive control(1 ng IL-6/ml) .

The results of this study indicate that partially degradable, poly(NIPAm/TB) particles efficiently captured and preserved IL-6 spiked in a complex biological fluid such as urine. Data confirmed that proteins captured by the particles and interacting with the high affinity molecular bait (in this case trypan blue) are protected from oxidative degradation. DHEA crosslinker degradation with NaIO4 resulted in increased hydrodynamic diameter of the particles and increased average pore size. This allowed the anti IL6 antibody to gain access to the IL6 antigen trapped inside the poly(NIPAm/TB) particles. In fact, the signal obtained from NaIO4 treated particles was 200% the signal obtained from untreated particles. Moreover, untreated particles yielded a signal that was equivalent to the ELISA background signal, therefore suggesting that untreated particles don't allow any significant interaction of the antibody with the trapped antigen whereas NaIO4 oxidizing treatment allows for antibody access to a preserved protein antigen. Another consideration to be made is that the interaction of the IL6 protein with trypan blue does not hinder antibody binding, thus suggesting that protein-trypan blue binding should take place in a region different from the antibody epitope and should not cause significant three dimensional conformations that alter antibody binding capacity. Mass spectrometry analysis of washing solution of poly(NIPAm/TB) particles that captured IL6 revealed no detectable trace of IL6, thus confirming that the interaction between IL6 protein and the trypan blue dye is very high and sufficient to retain IL6 in the particles through the washing procedure. Sensitivity of the test can be improved by increasing the amount of urine processed with the particles.

In order to have a sensitive and specific test for preeclampsia there is the need to evaluate panels of proteins associated to different biological functions. Proteins e,g, related to angiogenesis, inflammation, ischemia or invasion that can be associated to early events of placentation, along with marker for autoimmunity.can be investigated in the future and accompanied to IL-6 in the final form of the proposed A highly specific and sensitive preeclampsia will reduce the number of preeclampsia cases which are misdiagnosed or delayed and cause maternal or fetal mortality. Early diagnosis will be helpful in current medical practices for better management of the symptoms and decreasing health care cost.

Thus, future evaluation of the partially degradable nanoparticles for the integration in in vitro diagnostics IVD will be useful for the following reasons;

- Lab quality results within minutes (sensitivity and speed)
- Robust results (precision)
- High accuracy of predictions and reliability
- Least invasive for sample collection
- Integration with health care standard procedures

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BIOGRAPHY

Syeda Fatima Zaidi has been graduated from The University College of Pharmacy, Punjab University, Pakistan in 2009. She received her Master's of Science in Pharmaceutical Chemistry. She has worked as production Pharmacist at MASS PHARMA (pvt) ltd.