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Magnetic Purification Of Antibodies

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ABSTRACT

This work aimed at the development of magnetic nanoparticles for antibody purification and at the evaluation of their performance in Magnetic fishing and in a newly developed hybrid technology Magnetic Aqueous Two Phase Systems. Magnetic materials were produced by coprecipitation and solvothermal approaches. Natural polymers such as dextran, extracellular polysaccharide and gum Arabic were employed for coating of iron oxide magnetic supports. Polymer coated magnetic supports were then modified with synthetic antibody specific ligands,namely boronic acid, a triazine ligand (named 22/8) and an Ugi ligand (named A2C7I1). To optimize the efficacy of magnetic nanoparticles for antibody magnetic fishing, various solutions of pure and crude antibody solutions along with BSA as a non-specific binding protein were tested. The selectivity of magnetic nanoparticle for antibody, IgG, was found effective with boronic acid and ligand 22/8. Magnetic supports were then studied for their performance in high gradient magnetic separator for effective separation capability as well as higher volume handling capability.

The magnetic materials were also supplemented to aqueous two phase systems, devising a new purification technology. For this purpose, magnetic particles modified with boronic acid were more effective. This alternative strategy reduced the time of operation,maximized separation capability (yield and purity), while reducing the amount of salt required.

Boronic acid coated magnetic particles bound 170 ± 10 mg hIgG/g MP and eluted 160 ± 5 mg hIgG/g MP, while binding only 15 ± 5 mg BSA/g MP. The affinity constant for the interaction between hIgG and APBA_MP was estimated as 4.9×10^5 M^{-1} (Ka) with a theoretical maximum capacity of 492 mg hIgG adsorbed/g MP (Qmax). APBA_MPs were also tested for antibody purification directly from CHO cell supernatants. The particles were able to bind 98% of IgG loaded and to recover 95% of pure IgG (purity greater than 98%) at extremely mild conditions.

KEYWORDS: Magnetic nanoparticles, High gradient magnetic separation (HGMS), Aqueous two phase separation (ATPS), Antibody purification, Affinity ligand.

RESUMO

Este trabalho teve como objetivo o desenvolvimento de nanopartículas magnéticas para purificação de anticorpos e na avaliação do seu desempenho na pesca magnética e em uma tecnologia híbrida desenvolvida em combinação com sistemas de duas fases aquosas. Os materiais magnéticos foram produzidas por co-precipitação processos solvotérmicos, e posteriormente revestidos com polímeros naturais, tais como o dextrano, polissacarídos extracelulares e goma arábica. Os suportes magnéticos foram então modificados com ligandos específicos para anticorpos, a saber, o ácido borónico, um ligando de triazina (chamado 22/8) e um ligando de Ugi (chamado A2C7I1). Para optimizar a eficácia das nanopartículas magnéticas para a pesca magnética de anticorpos, foram testadas várias soluções de anticorpos puros e impuros, juntamente com BSA como uma proteína de ligação não específica. A selectividade das nanopartículas magnéticas modificadas com ácido borónico e ligando 22/8 foram as mais eficientes para a purificação de anticorpos. O processos de separação magnética foi avaliado quanto ao seu potencial para scale-up num separador magnético de alto gradient.

Os materiais magnéticos também foram adicionados a sistemas de duas fases aquosas, desenvolvendo assim uma nova tecnologia de purificação. Para este fim, as partículas magnéticas modificadas com ácido borónico foram mais eficazes. Esta estratégia alternativa reduziu o tempo de operação, maximizou a capacidade de separação (rendimento e pureza), ao mesmo tempo reduzindo a quantidade de sal requerido.

As partículas magnéticas revestidas com ácido borónico ligaram 170 ± 10 mg hIgG / g MP, eluindo 160 ± 5 mg hIgG / g MP, enquanto que a ligação a apenas 15 ± 5 mg de BSA / g MP. A constant de afinidade para a interação entre hIgG e APBA_MP foi estimado em 4.9×10^5 M^{-1} (Ka), com uma capacidade máxima teórica de 492 mg hIgG adsorvida g MP / (Qmax). APBA_MPs foram também testados para a purificação de anticorpos directamente a partir dos sobrenadantes de células CHO. As partículas foram capazes de ligar 98% de IgG e recuperar 95% de IgG puro (pureza superior a 98%) em condições extremamente suaves.

KEYWORDS: Nanopartículas magnéticas, separação magnética, Sistemas de duas fases aquosas, purificação de anticorpos, ligandos de afinidade.

TABLE OF CONTENTS

List of Figures:

List of Tables:

ABBREVIATIONS

Mabs –Monoclonal Antibody

BCA - Bicinchoninic acid

BSA - bovine serum albumin

MP-BA- Magnetic particles functionalised with boronic acid

DMAEMA – *N',N'*-dimethylaminoethyl methacrylate

DMF - *N,N*-dimethylformamide

DX - Dextran

FT-IR - Fourier transform infrared spectroscopy

HCl - Hydrochloric acid

hIgG - Human Immunoglobulin G

IgG - Immunoglobulin G

IDA - Iminodiacetate

Ka - Affinity constant

Ligand 22/8 - (2-3-aminophenol)-6-(4-amino-1-naphthol)-4-chloro-s-triazine

MNPs - Magnetic nanoparticles

NaOH - Sodium hydroxide

Qmax - Theoretical maximum capacity

SDS-PAGE - Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SEM - Scanning transmission electron microscopy

TEMED - *N,N,N',N'*-tetramethylene diamine

TEM - Transmission electron microscopy

HGMS- High gradient magnetic separator

ATPS- Aqueous two phase system

PEG- Polyethylene glycol

EPS- Extracellular polysaccharide

BACKGROUND

Monoclonal antibodies (Mabs) represent 20% of all biopharmaceuticals in clinical trials and are the fastest growing market. The major focus of biopharmaceutical industry nowadays is on high value added, low volume products with efforts to have less process operations and processing cost. In the near future, cost and capacity of therapeutics processing will become increasingly important.

The separation and purification of biopharmaceutical products are important factors from commercialization point of view based on customer demand and number of applications for treatment of diseases. High value biomolecules like proteins, antibodies and enzymes cost 70 % for their purification. Nowadays, Antibodies have become commercially important as drugs that are also generally called "biologicals" [1]. The challenging task in industrialisation of antibodies at larger scale is associated with development of cost effective and efficient processes. While many methods are now available for large-scale preparation of antibodies, crude products, such as cell harvests, contain not only the desired product but also impurities, which are difficult to separate from the desired product [2].

The important reason behind higher cost was associated with technologies that were used for processing of these products. Sensitivity of biomolecule plays important role from separation point of view which will affect usually antibodies, proteins and enzymes which are sensitive molecules for temperature as well as processing conditions responsible for making processing more difficult [3] . Biological sources such as cell culture conditioned media from cells expressing a desired antibody product may contain less impurity, in particular if the cells are grown in serum-free medium [4]. For the purpose to use the antibodies for disease treatment in human being higher purity standard is required which again make processing task more difficult. Various purification steps that were used contain application of low or high pH, high salt concentrations or other extreme conditions that may reduce the biological activity of a given antibody [5]. Thus, for any antibody purification it is a challenge to develop such a purification and separation technology which will provide higher purity as well as retain the biological activity of antibody. Those molecules that are not sensitive to processing conditions were easier to process and processing cost easily gets reduced [6]. During synthesis of these compounds by fermentation process they were produced in very less concentrations which contain highly mix composition of biomolecules, impurities and undesired components along with cells which needs to be removed [7]. Commonly used biomolecule purification technologies mainly involve primary process of capture of desired molecule from mixed culture broth in which huge volume was treated to eliminate higher level of impurities. In second step which is generally called as an intermediate step, in which antibodies are isolated from contaminants similar in size and/or physical/chemical properties, and finally a polishing step resulting in the high level of purity that is e.g. required from antibodies intended for therapeutic administration in human or animals [8]. Typically, the antibody purification steps are based on chromatographic separation of the compounds present in a given fluid [9]. Commonly used chromatographic techniques for small scale purification were

hydrophobic interaction chromatography, affinity chromatography and ion exchange chromatography. Improved development strategies were mainly associated with reduction of product residence time as well as downstream processing steps to enhance product yield and product quality [10]. With these strategies new trend come into existence which involves combination of multiple techniques to create hybrid technologies.

Commonly used method for antibody manufacture is cell culture method [11]. It is used to prepare antibodies for pharmaceutical or vaccine use in human population [12]. During the production using cell culture method various type of undesired components also generated or remain in the substrate as unused components. The various components that are present were proteins, carbohydrates, lipids and other molecules. These all impurities must be removed before use of antibodies for human diseases treatment. Widely used purification technique so far at laboratory scale and also at industrial scale is affinity chromatography [13]. Affinity chromatography involves use of Protein A or Protein G. The molecules used in affinity chromatography purification technique have capability to selectively bind antibodies from crude extract produced using cell culture technique. The antibodies that get bind to the affinity molecules can be easily removed from the binding surface by using suitable buffer solution [14]. The work efficient molecules like Protein A or Protein G were first covalently bound and packed by using agarose or sepharose beads and then filled in the column to carry out separation process effective and efficient. For operating of purification process using Protein A modified agarose filled column initially column is equilibrated with neutral buffer. After equilibration crude mixture containing from fermented cell culture is passed through the column. During this process of passage selective antibody molecules get bind to protein A molecules present in column while undesired components and contaminants pass out of the column [15]. Once crude sample passed through the column then it is again washed with neutral buffer solution and after that eluted using elution buffer to collect the desired antibody mixture [16]. Eventhough affinity chromatography is widely used it has several limitations associated with cost, purification capability, time consumption, processing capability, reusability, efficiency as well load handling capacity [17]. Because of above all limitations as well as low binding resin capacity is major bottleneck in use of affinity chromatography at larger industrial scale if economic and cost effective production of antibody is desired [18].

As advancement in research of cell culture technology leads to higher yielding titers which unables continuous processing to obtain higher productivity alternative to affinity chromatography is the need of future [19-20]. The importance of monoclonal antibodies as therapeutic medicine is due to the development of hybridoma technology and advancement of genetic engineering and bioprocess engineering. mAbs are commonly used for the treatment of cancer and autoimmune disorders having potential applicability for various disease control. The challenge of biotherapeutics are requirements in higher doses and productivity in g/L level. Because of low productivity and temperature sensitivity of

biomolecule downstream processing is not able to tackle productivity at larger scale. To achive mAbs having higher purity needs modification of purification process in terms of specificity, selectivity, reproducibility, product recovery, cost and storage stability. Common technologies that shows potential and capability to substitute conventional chromatography for mAbs purification involve application of affinity precipitation, membrane separation,expanded-bed chromatography, aqueous two-phase separation and magnetic separation.

Magnetic separation technique involve use of magnetic fluids which are colloidal dispersions having magnetic nanoparticles in suspension. These magnetic nanoparticles having surface functionalisation properties provide capability to adsorb desired biomolecule of interest. Due to smaller size magnetic particles able to develop dispersions which provide large surface areas per unit volume for effective separation and purification operation. The magnetic nanoparticle which is heart and basic building block of high gradient magnetic separation technique provide protein recovery from fermented broths. The high gradient magnetic separation process involve adsorption of a desired mAbs to magnetic nanoparticles, separation of the supports from magnetized filter and recovery of bound mAbs from MNPs. The magnetic separation process able to minimize the steps like filtration, membrane separation and centrifugation that are required with packed bed chromatography.

New type of hybrid separation and purification technologies plays important role in isolation of these types of biomolecules in higher purity and major research attention is provided by research community to develop much better and efficient techniques [21]. These small scale bioseparations are the most difficult and costly which is still biggest challenge for making these products cheaper and beneficial from customer point of view [22]. Hybrid process technologies provide advantages over conventional technologies in terms of persistent product quality through steady state operation, less impurities, reduction in product hold time leading to higher product quality, less intermediate steps, reduced capital cost as well as process volumes, higher speed with streamlined process and accelerated technology standardization, Product flexibility and compatibility with higher potential for productivity enhancement, Low cycle time with possibility for continuous operation [23]. PEG and Dextran has already been used in an aqueous two-phase extraction system which has been disclosed in the literature [24]. However the purity that is obtain using only ATPS is too low as It depends on many factors such as the concentration and molecular weight of phase forming polymers, the type and quantity of the salt and the type and concentration of additives (usually inorganic salts). Therefore, it is extremely difficult to find the appropriate aqueous two-phase extraction system for a given protein to be purified from a given source.

Still, ATPS represents a promising alternative for biological and chemical materials purification with tests already performed in industrial settings [25]. However, the time needed for phase separation and settling is high, the selectivity of the process is low and several unit operations can be required to achieve high purity. On the other end, the recycling of polymers and solutions used in ATPS is troublesome but needed due to the high costs involved.

Combination of ATPS with magnetic separation provide excellent process benefits as it minimize limitations of ATPS and incorporates benefits of magnetic purification. The association of ATPS with MNPs can not only improve the throughput of the process but also the selectivity of the separation. The entire research work of this thesis is directed specifically at antibody separation and purification but the results derived from this research work can be applied to various type of high-value biomolecules from market point of view.

References

1. Adriaenssens E.M., L. S. M., Vandersteegen K., (2012). CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. Virology, 434(2), 265-270.
2. Aguilar O. et. al. (2010). Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soyabean proteins. Sep. Sci. Technol., 45, 2210-2225.
3. An X., S. Z., Zeng H.,. (2003). Preparation of highly magnetic chitosan particles and their use for affinity purification of enzymes. Journal of Chemical Technology & Biotechnology, 78(5), 596-600.
4. Asenjo J.A. and Andrews B.A. (2011). Aqueous two-phase systems for protein separation: a perspective. J. Chromatogr. A, 1218(49), 8826-8835.
7. Bandeira V., P. C., Rodrigues A.F., (2012). Downstream processing of lentiviral vectors: releasing bottlenecks. Hum. Gene Ther. Method, 23(4), 255-263.
8. Barbosa H.S.C., H. A. V., Brocchini S., Slater N.K.H., Marcos J.C. (2010). Dual affinity method for plasmid DNA purification in aqueous two-phase systems. J. Chromatogr. A, 1217(9), 1429-1436.
9. Bensch M. et. al. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62, 2011-2021.
10. Bensch M., S. B., Hubbuch J,. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.
11. Bolognese B., N. B., Pico G.,(2005). Application of the aqueous systems of ethylene and propylene copolymer-maltodextrin for protein purification. J. Chromatogr B, 814, 347-353.
12. Bolton G.R. et. al. (2011). Addressing the challenges in downstream processing today and tomorrow. Biopharm Int., 24, S8-S15.
13. Chen J, T. J., Zhang Y, Wasserman A, Conley G, DiLeo M, Haimes E, Nixon AE, Ley A. (2010). The distinctive separation attributes of mixed-mode resins and their applications in monoclonal antibody downstream purification process. J Chromatogr A, 1217, 216-224
14. Chen S. et. al. (2009). A screening paradigm for the design of improved polymer-coated superparamagnetic iron oxide nanoparticles.Journal of Materials Chemistry, 19(35).

15. Chusainow J. (2009). A study of monoclonal antibody producing CHO cell lines - what makes a stable high producer? Biotechnol. Bioeng.,102, 1182-1196.
16. Ditsch A. et. al. (2005). High-Gradient Magnetic Separation of Magnetic Nanoclusters. Industrial & Engineering Chemistry Research, 44(17), 6824-6836.
17. Faria J. T., S. F. C., Converti A.,Passos F. M. L.,Minim V. P. R.,Minim L. A., . (2009). J. Chromatogr., B 877, 3031-3037
18. Frakas T., S. H., Tjerneld F.,. (1996a). Partitioning of bmannanase and a-galactosidase from Aspergillus niger in Ucon/Reppal aqueous two phase system. Bioseparation, 6, 147-157.
19. Gagnon P. (2008). The emerging generation of chromatography tools for virus purification. BioProcess Int, 6(S6), 24-30.
20. Gao J. et. al. (2012). Antibody affinity purification using metallic nickel particles. Journal of Chromatography B, 895-896, 89-93.
21. Gerster P., K. E. M., Hammerschmidt N.,. (2013). Purification of infective baculoviruses by monoliths. J. Chromatogr. A, 1290, 36-45.
22. Goloverda G. et. al. (2009). Synthesis of ultrasmall magnetic iron oxide nanoparticles and study of their colloid and surface chemistry. . Journal of Magnetism and Magnetic Materials, 321(10), 1372-1376.
23. Guan Y., L. T. H., Treffry T.E., Zhou C.L., (1996). Enzyme Microb. Technol., 19(6), 446.
24. Guoqiang D., K. R., Mattiasson B.,. (1994). Integration of aqueous phase extraction and affinity precipitation for the purification of lactate dehydrogenase. J Chromatogr A, 668, 145-152.
25. Heebol-Nielsen A., J. S., Hobley TJ.,Thomas ORT.,(2004). Superparamagnetic cation-exchange adsorbents for bioproducts recovery from crude process liquors by high gradient magnetic fishing. Sep Sci Technol, 39, 2891-2914.

CHAPTER 1

HGMS AND ATPS INTEGRATED HYBRID PROCESS TECHNOLOGIES FOR BIOPHARMACEUTICALS PURIFICATION

SUMMARY

Techcnological overview of processes that used in biopharmaceutical industry for purification and isolation of value added biochemicals which plays important role in prevention of serious human diseases with great market potential were illustrated in comprehensive manner. Todays industrial requirement mainly associated with process technologies which are cost effective, economical, as well as energy efficient and biomolecule friendly. Future requirement and consumer needs attract attention of researchers and scientist in biopharmaceutical industry towards hybrid and integrated process technologies that found promising to face challenges of new generation.

1.1 Introduction

The separation and purification of biopharmaceutical products are important factors from commercialization point of view based on customer demand and number of applications for treatment of diseases. Existing research work and methodologies represents that separation methods costs for 75 % [1-3] of the total production cost especially for products like proteins [4] , antibodies[5] and enzymes [6] The important reason behind higher cost was associated with technologies that were used for processing of these products [7-9] . Sensitivity of biomolecule plays important role from separation point of view [10] which will affect usually antibodies, proteins and enzymes which are sensitive molecules for temperature as well as processing conditions [11] responsible for making processing more difficult [12] . These molecules and its three dimensional structure get easily denatured [13] and loose its activity as well as applicability [14] by extreme pH [15], temperature [16] , pressure [17] ,solvents [18] as well as exposure to air [19].Those molecules that do not get damaged by above mentioned extreme conditions were easier to process and processing cost easily gets reduced [20]. Also from application point of view for clinical use these products needs to be in ultrapure form [21] . During synthesis of these compounds by fermentation process they were produced in very less concentrations [22] which contain highly mix composition of biomolecules [23], impurities and undesired components [24] along with cells which needs to be removed [25]. These impurities removal [26] , suspended solids separation [27] , product concentration followed by purification [28] is responsible for several bioprocessing steps which makes process more complex as well as costly [29]. Important components of bioseparation processes includes removal of cell components and fragments [30], product separation [31] and concentration, product purification to obtain higher purity product and polishing [32] which makes product acceptable for clinical and therapeutic applications [33].

Improved development strategies were mainly associated with reduction of product residence time [34] as well as downstream processing steps to enhance product yield and product quality [35]. With these strategies new trend come into existence which involves combination of multiple techniques to create hybrid technologies [36].Based on production and processing capacities bioprocesses were categorised in three main sections [37]. Processes whose process volume exceeds 100,000 L per batch and have superior separation strategies considered as 1arge-scale processes [38] . Products such as ethanol, polysaccharides, acetic acid and acetone are categorized in this type [39]. Medium scale processes involve antibiotics production [40] whose processing is quite robust and from cost point of view final cost depends on the initial volume reduction steps [41] . Small scale processes includes production of antibodies and enzymes [42] for therapeutic applications, usually fragile and delicate molecules are responsible for increase in separation and purification cost [43]. New type of hybrid separation and purification technologies plays important role in isolation of these types of biomolecules [44] in higher purity and major research attention is provided by research community to develop much better and efficient techniques. These small scale bioseparations are the most difficult and costly which is

still biggest challenge for making these products cheaper and beneficial from customer point of view [45]. This review involves illustration of high gradient magnetic separation and integrated hybrid process technologies formed with aqueous two phase extraction processes which will provide efficient and cost effective alternative to chromatography based as well as traditional non-chromatographic techniques.

1.1.1 Biopharmaceuticals purification considerations and market potential

In today's competitive market with large variety of products in developing stages as well as volatile market demand and tough competition from biosimilars [46] are responsible for biopharmaceutical companies to be under pressure to have efficient and cost effective solutions for market oriented manufacturing [47] . With this future challenging demand hybrid process technologies involving integration of multiple process techniques found to be promising [48].

Hybrid process technologies provide advantages over conventional technologies in terms of persistent product quality through steady state operation [49], less impurities, reduction in product hold time leading to higher product quality [50], less intermediate steps, reduced capital cost as well as process volumes, higher speed with streamlined process and accelerated technology standardization [51],Product flexibility and compatibility with higher potential for productivity enhancement [52], Low cycle time with possibility for continuous operation [53].

Leading class of biopharmaceuticals that attracts customers as well as have great potential from disease treatment point of view, involves monoclonal antibodies [54] which will be one of the important constraints from disease treatment point of view. The biopharmaceuticals that are in the market with their applications are illustrated in Table-1.1.

1.2. Traditional high gradient magnetic separation

High gradient magnetic separation (HGMS) technique involve use of ligand modified superparamagnetic particles for selective adsorption of biomolecules from crude mixture followed by separation using magnetic field and subsequent elution with buffer solution [55]. The magnetic particles have common size range from 500 nm to 3000 nm and are non-porous in nature. HGMS initially come into existence as an alternative for biomolecule separation from high viscosity mixtures where high speed centrifugation and membranes are less attractive options [56]. During recent year's developments in ligand modification extends the applications of magnetic particles for purification of biomolecules from highly complex crude biological mixtures[57] . Table 1.2 illustrates different types of particles used for biopurification application. At present, nanosized magnetic particles obtain using Fe_3O_4 attracts attention of researchers for bioseparation as it provides higher surface area to volume ratio [58]. Inspite of several advantages use of magnetic particles for large scale applications remain limited because of cost constraints. The commercially available particles have higher cost hence they should be used wisely in order to have more economic purification [59]. With this need integration of magnetic particles with other technologies to make separation in cost effective manner will be suitable option. These fusion technologies are called as hybrid processes and are attracting popularity because they provide benefits which are difficult to obtain using individual technique.

Table 1.1: FDA approved biopharmaceuticals in the market and their therapeutic applications

Year	Company	Brand Name	Disorder Treatment	Target
2014	Takeda	Vedolizumab	Crohns disease and ulcerative colitis	Integrin antagonist
2014	Biogen Idec	Eloctate	Hemophillia A	Coagulation factor VIII
2014	Biogen Idec	Alprolix	Hemophillia B	Coagulation factor IX
2014	Santarus	Ruconest	Hereditary angioedema	C1 activation inhibitor
2013	Bayer	Riociguat	Pulmonary hypertension	Guanylate cyclase stimulator
2013	Pfizer	Palbociclib	Breast cancer	Kinase-4/6 inhibitor
2013	Biogen Idec	Obinutuzumab	Chronic lymphocytic leukemia	CD20
2013	Genzyme	Lemtrada	Multiple sclerosis	CD52
2012	Medivir	Simeprevir	Hepatitis C	Protease inhibitor
2011	Actelion	Macitentan	Pulmonary hypertension	Tissue targeting endothelin receptor antagonist
2009	Roche	Actemra	Rheumatoid arthritis	IL-6
2008	Centocor	Stelara	Psoriasis	IL 12 and IL 23
2008	Centocor	Simponi	Rheumatoid arthritis	TNFα
2007	Alexion	Soliris	PNH	C5 Complement
2006	Amgen	Vectibix	Colorectal cancer	EGFR
2004	Bristol-Myers	Erbitux	Colorectal cancer	EGFR/Her1
2004	Genentech	Avastin	Colorectal cancer	VEGF
2004	Genentech/Xoma	Raptiva	Psoriasis	CD11a
2004	Biogen-Idec/Elan	Tysabri	Multiple sclerosis	A4 integrin
2003	Corixa/GSK	Bexxar	Non-Hodgkins Lymphoma	CD20
2003	Genentech/Novartis	Xolair	Allergy	IgE
2002	Biogen-Idec	Zevalin	Non-Hodgkins Lymphoma	CD20
2002	Abbott	Humira	Rheumatoid arthritis	TNFα
2001	Takeda	Campath	B cell chronic lymphocytic leukemia	CD52
2000	Wyeth-Ayerst	Mylotarg	Acute mylogenous lymphoma	CD33
1998	Novartis	Simulect	Prophylaxis of acute organ rejection	IL2R
1998	Medimmune	Synagis	Respiratory Synctial Virus	RSV
1998	Centocor	Remicade	Rheumatoid arthritis	TNFα

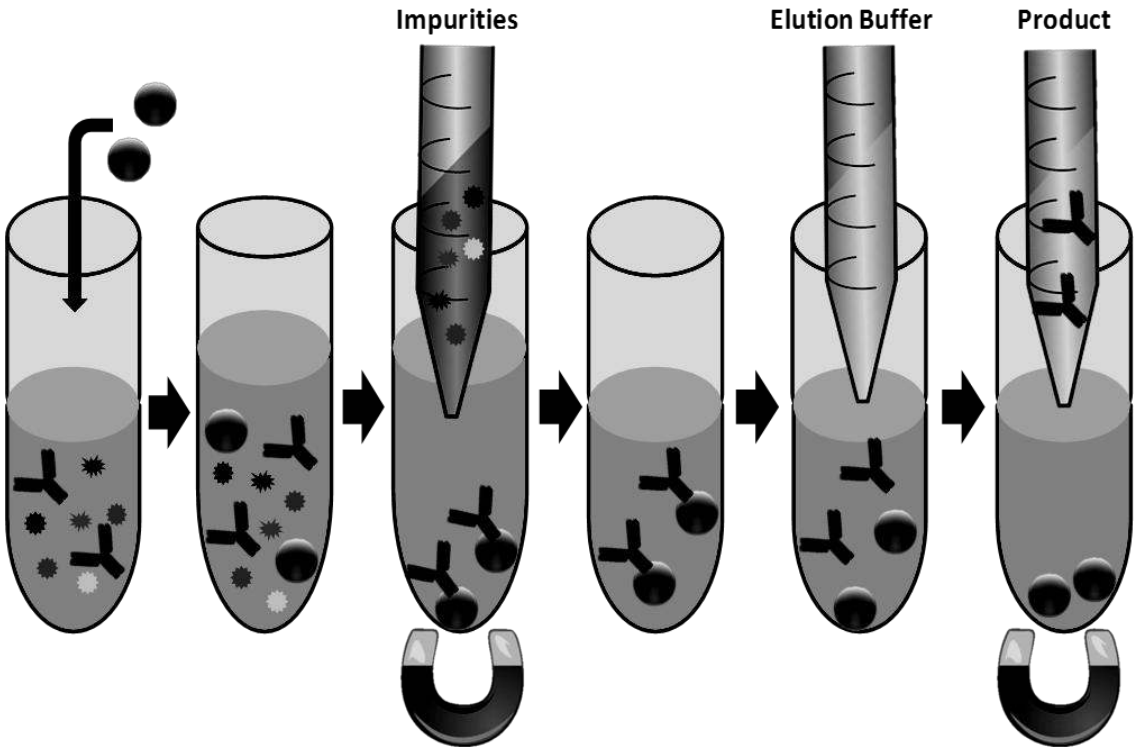

Figure 1.1: High gradient magnetic separation process schematic outline

1.3. Traditional Aqueous Two Phase System (ATPS)

Simplified form of aqueous two phase systems are obtained by mixing two water soluble polymers or polymer and salt. Preferred polymers for simple ATPS are polyethylene glycol and dextran to form polymer-polymer system. Simplified form of aqueous two phase systems are obtained by mixing two water soluble polymers or polymer and salt. Preferred polymers for simple ATPS are polyethylene glycol and dextran to form polymer-polymer system [60]. In alternative cheap systems dextran can be replaced by salts like citrate or phosphate to form polymer-salt system. Inspite the popularity of technique it is used frequently at research scale only because of final product purity constraints; hence it needs to be integrated with other processes which will enhance purity as well as product recovery. Non- functionalised ATPS provide suitability for certain type of biomolecules as illustrated in table [3].

The important parameter which plays vital role in ATPS includes molecular weight of polymer, temperature, pH and electrolyte concentration. For industrial scale applications important constraints are capability to provide higher purity at affordable cost. Integration of smart polymers, magnetic nanoparticles, precipitating agents, surfactants, free ligands and protein conjugates have capability to fulfil these requirements with possible large scale applications.

Table 1.2: Performance of ligand modified magnetic particles in biomolecules purification

Magnetic particle type	Biomolecule	Source	Yield (%)	Purity (%)	References
MNP-Boronic acid	Human Immunoglobulin-G	CHO	67	74	[27]
MNP-NH_2-Protein A	Mouse Ig2b	Mouse Serum	75	----	[47]
MNP-NH_2-Lysozyme	Fv antibody fragment	E.Coli	53	90	[137]
MNP-NH_2-Benzamidine	Trypsin	Porcine pancreas	62	----	[49]
MNP-Glutaraldehyde-polymethacrylate	Mouse IgG 2a	Mouse ascites	58	71	[72]
MNP-NH_2-sulfur trioxide	Lactoferrin	bovine whey	47	26	[78]
MNP-PEG-MEHDE	Human Immunoglobulin-G	Human plasma	----	73	[88]
MNP-NH_2-Dextran	Lectin	Jack beans	69	98	[78]
MNP-Poly(styrene-vinyl acetate-diivinylbenzene)	Human Immunoglobulin-G	Human serum	54	78	[99]
MNP-NH2-IDA	Dismutase	bovine whey	79	----	[78]
MNP-polyvinyl alcohol-anti-IFN-α-2b IgG	Interferon α-2b	Pseudomonas cell lysate	51	56	[99]
MNP-Poly(VADB)-2 mercaptonicotinic acid	Human Immunoglobulin-G	Human serum	80	52	[38]
MNP- Chitosan	Trypsin	Bovine pancreas	73	----	[4]
MNP-NH_2-sulfur trioxide	Lactoperoxidase	bovine whey	90	----	[46]
MNP- NH2	Lactoperoxidase	bovine whey	90	----	[134]

1.4. Emerging high gradient magnetic separation and aqueous two phase systems

1.4.1 Temperature sensitive polymer integrated ATPS

Commonly used polymers for aqueous two phase extraction are polyethylene glycol and dextran but the major disadvantage of this technique is complexity in biomolecule separation from phases [61]. With the help of temperature change responding polymers it is possible to make biomolecule purification

easier and effective [62]. These polymers perform phase separation by externally increasing temperature above polymers cloud point. The complex formed between biomolecule and polymer gets partitioned to top phase which is then separated using temperature change effect [63] . By heating the polymer above lower critical solution temperature (LCST) thermo responsive polymer can easily separated from water solution [64] . The common purification steps includes initial partitioning of two polymers in a system composed of two polymers, adjustment of medium conditions which will displace target biomolecule to thermoresponsive polymer phase.This temperature change results in formation of new two phases in which one is water buffer phase having biomolecule partitioned and other contains concentrated polymer rich phase which is recovered and recycled [65]. Thermosensitive polymer modified ATPS examples are tabulated in table [4].

Table 1.3: Performance of traditional ATPS in biomolecules purification

Biomolecule	ATPS	Source	Yield (%)	Purification factor (PF)	Purity	Reference
Recombinant Bacillus haloduransxylanase	PEG/ Phosphate	E.coli	92	----	48	[100]
Human antibodies	PEG/ Phosphate	CHO	76	----	55	[108]
Amylase	PEG/Citrate	----	80	2	----	[135]
Elastase	PEG/Phosphate	----	89.5	----	60	[9]
human Immunoglobulin G	PEG/Phosphate	CHO	88	4.3	----	[131]
mAb 2G12	PEG/Phosphate		----	2.01		[94]
Human interleukin	PEG/Sulphate	CHO	98	2.3	86	[38]
Proteases	PEG/Citrate		97	4.2		[96]
hIgG-anti-HIV	PEG/Phosphate	Transgenic tobacco extract	95	3-4	----	[93]
Immunoglobulin G	PEG/Dextan	Hybridoma cells	96	----	78	[7]
Pectinase	PEG/Na_2SO_4	Plant origin	90	2.5		[4]
Immunoglobulin G	PEG/Citrate	Hybridoma cells	99	3.3	65	[8]
Human interferon α1	PEG/Phosphate ester	E.coli	76	----	25	[43]
Aspergillopepsin I	PEG/NaH_2PO_4	Aspergillus niger	99	5	73	[90]
Penicillin acylase	PEG/Citrate	E.coli	80	5.5	48	[75]
Xylose reductase	PEG/Sulfate	E.coli	97	3.1	72	[31]
LectinConGF	PEG/Citrate	----	99	4.8	----	[96]
Thaumatin	PEG/Salt	----	96	3.7	----	[3]
Hepatitis B antigen	PEG/Phosphate	Yeast cells	89	3.5	----	[124]

In case of thermosensitive polymer system ATPS were formed using both or one polymer as thermosensitive and using one or more extraction enhancer [66]. In both type of system it is possible to recycle and reuse thermoresponsive polymers by using phase separation induced by temperature. Using both polymers as thermo responsive is modified form of the system in which only one thermo responsive polymer is used along with mixed ATPE of common ingredients.

Thermosensitive polymer like ethylene oxide-propylene oxide used as top phase forming polymers whereas dextran or starch derivatives form bottom phase [67]. Target biomolecule is first partitioned to EOPO phase which is then isolated and temperature is raised above EOPO polymer cloud point temperature responsible for formation of polymer phase and water/buffer phase containing desired biomolecule.

In other type of ATPS system in which both polymers are thermosensitive, prepared by using EO50PO50 and HM-EOPO [68] . EO50PO50 is a random copolymer of 50% ethylene oxide and 50 % propylene oxide, where as HM-EOPO is a random copolymer of EO and PO with aliphatic C14H29 groups coupled to each end of the copolymer. These both polymers in a water solution at a certain temperature form a two phase system in which the top phase get depleted in polymer whereas bottom phase get enriched [69].

Propylene oxide concentration has impact on cloud point as rise in its concentration decreases the cloud point. For HM-EOPO concentration dependence on the cloud point temperature was stronger as compared to EO50PO50 polymer. Because of this dependence phase composition for HM-EOPO/water system has more impact compared to EO50PO50/water system at the time of temperature change for thermoseparation [70). The presence of alkyl end group of HM-EOPO also gives polymer surfactant like properties responsible for formation of micellar aggregates. Repuslive forces between HM-EOPO and EO50PO50 is responsible for phase separation which cause EO50PO50 to form top phase and HM-EOPO to form bottom phase. In comparison with EO50PO50/dextran system phase separation in HM-EOPO/EO50PO50 system occurs at lower concentration.[71] illustrate the thermoresponsive system which incorporates UCON/ dextran /TEG-COOH in a composition of 8% / 6% / 20% which provides 85% recovery with a total purity of 88 %. Because of thermoseparating property of TEG-COOH it is easier to separate the polymer and recycle effectively. Other polymers that found suitability for this technique includes poly (N-isopropylacrylamide), polyvinylcaprolactum, ethyl (hydroxyethyl) cellulose, poly (N-vinylcaprolactam-covinyl) imidazole.

Table 1.4: Temperature sensitive polymer ATPS for biopharmaceuticals purification

ATPS	Biomolecules	Source	Yield (%)	Purification factor (PF)	Purity	Reference
EOPO/Waxy barley starch	Cutinase	Fusarium solani pisi	71	2.5	83	[61]
EOPO/HM-EOPO	Apolipoprotein A-1	E.Coli	66	6	----	[55]
EOPO/Dextran	hIgG	CHO	85	----	88	[32]
UCON/ Reppal	α-galactosidase	Aspergillus niger	62	4.7	84	[33]
Reppal/EOPO	Apolipoprotein	E.Coli	82	3	----	[3]
PEG-(COOH)2 / Dextran	hIgG	CHO	82	3.6	96	[10]
UCON/ dextran /TEG-COOH	hIgG	CHO	76	----	85	[32]
UCON/ benzoyl dextran	endo-polygalacturonase	E.coli	57	----	91	[91]
EOPO/Starch	Human growth hormone	E.Coli	70	5	----	[46]
EOPO/Maltodextrin	Lysozyme	E.coli	81	3	80	[19]
EOPO/Ammonium sulphate	α-Amylase	E.coli	73	4.5	78	[69]

For enzymes purification thermally responsive polymers systems which show effective phase separation properties in the temperature range 20°C – 25°C found to be highly beneficial. The system composition includes Ucon 50-HB-5100/polyvinyl alcohol (Mw 10,000), Ucon 50-HB-5100/$(NH_4)_2SO_4$, Ucon 50-HB-5100/starch modified with hydroxypropyl [72]. The systems which provide higher biomolecule concentration as well as maintain higher activity are illustrated by [73] . The process mainly focused on purification of endo-polygalacturonase providing concentration upto 10 times concentration with activity above 95%. [74] studied partitioning of α-amylase in systems such as polyethylene oxide-polypropylene oxide/ammonium sulphate.

1.4.2 Chemically modified polymers and free ligands for enhanced biomolecule purification

Biomolecule purification can be enhanced in aqueous two phase system using chemically modified polymers which have better affinity for desired components [75] . The polymers that posses property to be chemically modified using specific chemical groups to have better biomolecule purification capabilities are polyethylene glycol and triethylene glycol. These both polymer molecules can be easily modified using mercaptoethyl pyridine (MEP), aminoquinuclidine (AQ1), pyrimidine, glutaric acid, sulfonates as well as triazine based groups [76].

In association with chemical modification of polymer, free ligands also incorporated in aqueous two phase system. Those ligands that have possibility of incorporation in aqueous two phase system are either of biological origin or synthetic origin [77] . Commonly used biological ligands are protein A

produced using Staphylococcus aureus and Protein G produced in group C and group G streptococci having strongly binding capacity to Fc region [78]. Other type suitable in this category are Protein L, Protein P, Protein D synthesized in Peptococcus magnus, Clostridium perfringens and Branhamella catarrhalis respectively. Protein L and P binds k light chains where as protein D binds to IgD. The light chain binding property of Protein L and P make them appropriate for purification of engineered antibody fragments [79] .

1.5 Hybrid process technologies

1.5.1 HGMS integrated with fermentation

Insitu magnetic fishing with fermentation joins multiple unit operations in single step by reducing processing cost and having product recovery much easier. In this process ligand modified magnetic particles were added in bioreactor in which cells are growing and producing biomolecule of interest which is continuously trapped by functionalised magnetic particles. For successful operation of this process cells should be able to acclimatize with magnetic particles environment in order to function in superior natural way to have better performance and higher yield. Magnetic particles used for this process should be shear resistant as shear force if particles get break due to shear they will transform into extreme fine which will interfere with cell metabolism causing difficulties in growth as well as productivity. Other important properties that magnetic particle should posses consists non-porous nature for biomolecule to be purified [80]**.** If particle has porous nature biomolecule will be trapped in pores and it will be difficult to elute them out using elution buffer [81]. The biomolecule binding specific area of magnetic adsorbent should be higher to capture higher amount of biomolecule as possible [82] . Magnetic particles should disperse easily in media mixture to have better contacts for fishing out of biomolecule. Particles should posses higher magnetic property so that they can be easily separated using external magnetic field from complex media [83]. The particles should have superparamagnetic behaviour in order to get magnetised easily but as soon as magnetic field get removed it become free from magnetic field. Absence of magnetic property after removal of magnetic field helps in easy redispersion of magnetic particles for superior elution conditions [84]. Magnetic force that is exerted on the particle in high gradient magnetic separator mainly depends on volume of particles, particle magnetization and gradient of magnetic field strength [85].

1.5.2 ATPS integrated with fermentation

Extractive fermentation put forward one challenging approach which involves continuous fermentation with simultaneous product recovery using aqueous two phase extraction system. The important goal of this technique is to avoid inhibition of fermentation process caused due to accumulation of final product of interest. In this approach ATPS is used as integrated step to remove product from the fermentation broth simultaneously during its production. The final product inhibition can be easily

prohibited as product is removed continuously. The work done by Jiang et.al. [86,87] shows purification potential of L-asparaginase from E.coli. fermentation broth using PEG/Phosphate system. Nisin was purified from lactococcuslactis using PEG 20000/$MgSO_4.7H_2O$ by [88,89]. Chavez-Santoscoy A. [90] used extractive fermentation technique for recovery of β-carotene from synechocystis sp. PCC 6803. They able to recover 95.9% β-carotene using PEG/Dextran system. [91] shows capability of technique for monoclonal antibody production using hybridoma and CHO cells.

Process use polymer-polymer system in which cells were grown in bottom phase and product (IgG) was recovered in top phase. Similar studies from [92] and [93] shows use of technique for other biomolecules like Cephalexin, Xylanase, 6-phenyl-α-pyrone from fermentations of immobilized Penicillin G acylase, E.coli and Trichodermaharzianun. ATPS used in these systems were PEG 600/ammonium sulfate, PEG 6000/Phosphate and PEG 8000/Sulfate respectively.

1.5.3 High gradient magnetic separation integrated with ATPS

Thrust of biopharmaceutical industry to have novel, low cost and easy to scale up non-chromatographic methods for the downstream processing of antibodies is responsible for attracting attention from the academic as well as industrial communities towards aqueous two phase extraction (ATPE) and magnetic fishing as a interesting alternatives to chromatographic methods. Aqueous two phase extraction is such a versatile technique that it can be easily coupled with magnetic nanoparticles [94].

Research work carried out by our group demonstrates the potential to combine aqueous two-phase extraction (ATPE) with magnetic separation for developing a non-chromatographic and efficient method for the antibody purification from cell culture supernatants [95]. Aqueous two-phase systems (ATPS) consisting of polyethylene glycol (PEG Mw 3350) and dextran (Mw 500,000) was integrated with boronic acid functionalized magnetic particles in association with salt of different concentrations ranging from 100 mM to 500 mM. Extraction studies were carried out by through mixing of all components followed by phase segregation and particle separation. The isolated particles were eluted using elution buffer in order to have desired antibody recovery. The particles used in study were again regenerated and reused for more than five times in order to make process more beneficial and cost effective. Partitioning of biomolecule in integrated hybrid technique is influenced by complex phenomena which involve van der Waals forces (Luechau F., 2011), hydrogen bonds (Bensch M. et. al., 2007), charge interactions [96-98], hydrophobic interactions and steric effects [99-101].

1.5.3.1 Magnetic ATPS and performance of polymer coated particles

Coating of magnetic particles with various types of polymers for evaluation of performance as well as partitioning effect is one of the important topic of study; it also provides important information regarding suitability of polymer for specific aqueous two phase extraction system. The primary reason behind magnetic particle coating is associated with magnetic core protection from harsh processing conditions,

as well as improving biocompatibility and colloidal stability [102-104]. The performance of polymer coated magnetic particles in ATPS was studied by our group. The polymers studied were dextran-500000, gum arabic, carboxymethyl dextran, PEG- 3500, EOPO, jeffamine-M-2070, polyacrylic acid sodium salt-15000, UCON-2000 and UCON-3520.

For polymer coating study purpose, MPs coated with the selected natural and synthetic polymers with distinct properties, were prepared and then supplemented to PEG/Dextran ATPSs, followed by investigation of IgG partitioning in the upper and lower phases as well as binding to particles at different salt concentrations (100, 200, 300, 400 and 500 mM NaCl) [105-108]. Increase in salt concentration decreased the IgG concentration in the lower dextran phase with simultaneous rise of IgG concentration in the upper PEG-rich phase, particularly for salt concentrations greater than 200 mM. The rise of salt concentration also decreases non-specific ionic interactions and enhances hydrophobic interactions which promote the transfer of IgG between phases [109] . On the other hand, the amount of IgG adsorbed onto polymer coated MPs (which usually partition to the bottom phase) tend to increase with the increase in salt concentration. This effect is particularly evident for EOPO, PEG, jeffamine and polyacrylic acid coated MPs. Particles coated with UCON and carboxymethyl dextran yielded the highest amounts of human IgG bound, for all salt concentrations tested, as opposed to the dextran and gum arabic coated MPs (DX-MP and GA-MP, respectively) which bound the lowest amounts of human IgG.

Advantages of Magnetic Aqueous two phase extraction technology

The technique illustrated in article (Dhadge V.L., 2014) has special advantages in comparison with ATPS or direct magnetic fishing alone in terms of increase of yield and purity of the target substance recovered from complex crude mixtures, enhancement of mixing and reduction of phase separation time with application of external magnetic field, reduction in magnetic material requirement, selective partitioning of target antibodies with less salt utilization in partitioning and purification of substance, use of robust versatile as well as cheap magnetic material produced by a wide variety of methods having effective pH resistance, easy interaction reversibility between target substance and magnetic material for product recovery, compatibility with wide variety of solvents and solutions, removal of surfactant requirement need, provides possibilities of utilization for various type of magnetic fields which will allow easier adaptation and optimization of the process.

Table 1.5: Comparison of the hybrid process described with direct magnetic fishing and ATPS

Type of system		Yield (%)	Purity (%)	Amount of MPs needed per ml of crude	Time required for phase separation in ATPE (min)	Time needed for incubation (min)	Ligand attached to MPs	Mg of Salt per gm ATPE	Eluent Buffer
Direct magnetic fishing (Dhadge V.L., 2013)		67	74	10 mg	NA	15	Boronic Acid	NA	Tris-HCl (pH-8.5)
Direct magnetic fishing (After three cycles) (Dhadge V.L., 2014)	Cycle 1	38	90	20 mg	NA	15	Ligand-22/8	NA	Glycine-NaOH (pH-11)
	Cycle 2	13	88	Reuse	NA	15	Ligand-22/8	NA	Glycine-NaOH (pH-11)
	Cycle 3	7	85	Reuse	NA	15	Ligand-22/8	NA	Glycine-NaOH (pH-11)
	Total	58	87	20 mg	NA	15	Ligand-22/8	NA	Glycine-NaOH (pH-11)
ATPE system alone In one step extraction		23	39	NA	40	NA	NA	150 mg	NA
Hybrid process (Dhadge V.L., 2014)		92	98	5 mg	25	10-30	Boronic acid	11.6 mg	Tris-HCl (pH-8.5)
Hybrid process (After two cycles)	Cycle 1	60	98	10 mg	25	5-40	Ligand-22/8	11.6 mg	Glycine-NaOH (pH-11)
	Cycle 2	30	92	Reuse	25	5-40	Ligand-22/8	NA	Glycine-NaOH
	Total	90	95	10 mg	25	NA	Ligand-22/8	11.6 mg	Glycine-NaOH (pH-11)

1.5.4 Magnetic adsorbents integrated with micellar ATPS

Magnetically enhanced micellar ATPS are formed by dissolving specific type of surfactants in water with incorporation of magnetic particles. Micellar ATPS split into two distinct phases when temperature increased or reduced in relation to cloud point temperature. The phases that get formed are one surfactant rich phase and other surfactant weak phase. Advantage of micellar ATPS are associated with easiness of recycling of phase forming polymers [110-113] because there is need to process only surfactant rich phase. Micellar ATPS are extensively studied specially for extraction of hydrophobic proteins. After incorporation of magnetic particles in micellar ATPS utilisation of external magnetic field is possible for accelerated phase separation.

Micellar extraction with magnetic particles considered as one of the efficient as well as economically viable process. Micelles were formed spontaneously by clustering of polar head groups of surfactants around inner core of water or magnetic particle above critical micelle concentration (CMC) [114]. Micelles are nanometer scale aggregates and are thermodynamically stable. Noncovalent aggregations of individual surfactant monomers are responsible for formation of micelle [115]. Micelles have spherical, cylindrical or planer shape which is easily controlled by changing solution conditions, temperature, ionic strength, pH, surfactant concentration and composition [116-119].

Surfactant head plays important role in micelle growth requires bringing the surfactant heads closer to reduce the area at micelle surface [120-122]. Micelle formation is an outcome of balanced intermolecular forces, such as electrostatic, hydrophobic, steric, van der Waals interactions as well as hydrogen bonding [123]. Micelle gets formed when attractive and repulsive forces balance each other. Non polar surfactant tails are responsible for hydrophobic effect and hence attractive force whereas steric and electrostatic interactions are responsible for repulsive force. Change in pH or temperature of magnetic micellar system is responsible for phase separation in micelle rich and micelle poor phase. Internal energy and entropic changes are the main reasons behind phase separation. Important task in use of modified ATPS with magnetic particles depends on finalising operating conditions. Use of ligand modified magnetic particles in micellar ATPS enhances yield of biomolecule purification as well as final purity of product [124-126] .

1.5.5 Aqueous two phase extraction integrated with Affinity precipitation

Incorporation and coupling of affinity precipitation with aqueous two phase extraction joins the benefits of both techniques as well as eliminate limitations making it more effective from purification point of view [127]. Even though affinity precipitation method was known for more than 30 years it recently received more attention because of investigation of new type of materials [128]. Usually precipitation occurs by changing physical or chemical parameters which transform soluble substance in solution to insoluble form. Traditionally commonly used precipitation agents were Ammonium sulfate, trichloroacetic acid, sodium deoxycholate and caprylic acid [129]. Ammonium sulfate is widely used in this field because of simplicity, provides rapid effect as well as easy to use. Combination of affinity precipitation with aqueous two phase extraction benefits by effective cell debris removal using extraction followed by precipitation .Precipitation step is responsible for recovery of desired biomolecule as well as precipitating compound. The important properties that polymers should posses in order to have effective hybrid affinity partitioning with ATPS are associated with availability of reactive group for coupling of ligand, negligible interaction with impurities to prevent non-specific precipitation of impurities, easier phase separation on change of medium property, capability to form compact polymer precipitates, should avoid trapping of impurities in precipitate, easier precipitate solubilization property, polymer should be cheap and must provide higher product recovery as well as purity. The hybrid process steps includes mixing of crude extract to be purified with ATPS forming polymers, mixing of the components followed by centrifugation, removal of top phase, addition of biomolecule conjugate forming component in top phase with incubation for 15 minutes at room temperature, lower the pH to induce precipitation of conjugate, centrifuge to remove precipitate, solubilization of precipitate and biomolecule recovery.

In this integrated process partitioning of biomolecule is directed to top phase of ATPS prepared using polymers like polyethylene glycol and modified starch such as Reppal PES 200 or dextran. The undesired components and proteins along with cell fractions partition into lower phase. Precipitation of desired biomolecule and polymer complex is triggered by either pH change or temperature or by cation addition. Based on this integrated technique [130] carried out lactate dehydrogenase and protein A purification using a conjugate of Eudragit S-100 with the triazine dye Cibacron blue, Chitinase was effectively purified by S.Teotia et al [131] using Chitosan and polyethylene glycol / phosphate system. In this method they incorporate chitosan to form conjugate with Chitinase which was distributed to PEG phase. Top phase containing Chitosan-Chitinase conjugate was removed and precipitated by adjusting pH. Hybrid ATPS consisting of polyethylene glycol (Mw 6000) and Reppal PES 200 was also used by Jhy-Ping Chen et.al. [132] to purify trypsin using conjugate formed by soyabean trypsin inhibitor with hydroxylpropyl methylcellulose acetate succinate. The process also proves its potential of purification for pullulanases, α-amylases, xylaneses and lectins from plant as well as animal sources.

1.5.6 ATPS integrated with Solvent Sublation (Aqueous two phase floatation)

Solvent sublation technique is based on principle of adsorptive bubble separation. Integration of this technique with aqueous two phase extraction provides hybrid process that can be effectively used for penicillin G from fermented broth. Important steps of process involve initial adsorption of biomolecule of interest on bubble surface generated by inert gas passing upward in process vessel. The biomolecules adsorbed on the bubble surface were then collected using polymer layer which is situated at the top.

For preparation of process initially crude mixture containing biomolecule of interest was mixed with salt like ammonium sulfate to form mixture and then PEG solution was added to develop organic layer on top of the mixture. Neutral gas like nitrogen is used to sparge from bottom of process vessel to form bubbles that pass from bottom phase to top PEG rich phase. Bubble adsorb biomolecules of interest and carries with it which get break in top PEG rich phase releasing adsobed molecule in that phase.

Process has advantages of easy operation, less solvent requirement, high product concentration and less time requirement. In related approach Li et al. [133] show process application for linomycin separation and purification.

The important process control parameters are salt concentrations, PEG concentration, gas floatation time and flow rate. Bi et. al [134] shows that process shows better outcome as compared to individual aqueous two phase extraction as well as solvent sublation. Process shows higher yield in terms of superior product yield which will accelerate the yield to have much better efficiency. The product purity can be accompanied by using superior product yield which will be accounted as basic change out for superior qualities. The product intensity will be used to stabilize the challenges to have major outcomes from the product intensity point of view. Higher product intensity can be utilised when considering higher values of purity as well as yield.

1.5.7 Hybrid monoliths

Monoliths are network of interconnected channels, casted as a single fixed bed. The advantages of monoliths are higher as well as effective flowrates with uniform distribution of packed particles beds and convective mass transport [135]. Developments in molding process based rigid macroporous polymer monoliths finds its way in various fields mainly bioprocess, biopharmaceuticals, food and tissue engineering. Monoliths also proves their possibility to be used as bioreactors and shows integration capacity with variety of chromatographic techniques such as affinity chromatography, hydrophobic interaction chromatography, ion exchange chromatography, high performance membrane chromatography as well as capillary electrochromatography [136]. At present situation monoliths find their applicability in gene therapy and in the field of vaccination for purification of proteins, viruses and plasmid DNA. Monoliths can be used as column packing material by inserting in a column, as they have capacity to densely occupy the column volume. Monoliths also show superior engineering properties like low mass transfer resistance and low back pressure. The specific size and shape of monolith is responsible for

convective mass transport which avoids diffusion flow [137] .The work done by Barroso et.al. shows successful integration of magnetic nanoparticles in monoliths which plays important role in terms of enhancing applications from purification point of view. Hybrid monoliths can be successfully used for purification of monoclonal antibodies which are one of the highly important segments of biomolecule from market point of view.

Hybrid monoliths improve purification process with simple implementation and production. These supports are economical from manufacturing prespective as well as provide higher productivity for certain demand. Utilisation of hybrid monoliths in chromatographic processes needs control of several properties to have superior purification capability. The material used for synthesis of monoliths play important role in order to have superior properties along with parameters and preparation methods.

Hybrid monoliths can be easily assessable to higher flow rate and provide steady state performance without loss of activity. The advantage of easier adaptability of higher flow rate is associated with independence of binding capacity from residence time. One important advantage of hybrid monoliths is that it does not have void volume which will eliminate turbulence in the system of use, as turbulence is responsible for shear in a column because of presence of magnetic particles. If small size hybrid monoliths with higher porosity are used with higher flow rates it will allow working at low operating pressure. Basic material of monolith synthesis is either organic or silica based, which are prepared by sol-gel process leading to formation of better interconnections with better permeability and mechanical stability. In case of organic polymer based monoliths incorporating magnetic particles commonly used materials are chitosan, polymethacrylates, and polyacrylamides. For preparation of hybrid monoliths chitosan used is obtain using a biodegradable polysaccharide extracted from crustacean shells called as chitin. The polymer has certain advantages as environment friendly and biocompatibility which acts as better material for biopharmaceuticals purification. Hybrid magnetic monolith synthesis consists of use of monovinyl monomers, crosslinking agent, magnetic particles, initiator and pore-forming solvent. Type of polymerization used is a radical polymerization and is carried out using either ultraviolet irradiation or heat. The monomers that are used for hybrid monoliths preparation provide mechanical stability to monolith whereas magnetic responsive nature of magnetic particles enhances monoliths performance.

Method used for hybrid monolith synthesis also plays important role for having specific characteristics. The most preferred method is freeze-drying which provides better interconnectivity inside the monolith structure. Traditional monoliths that are marketed by several companies are convective interactive media prepared using polymethacrylate having pore size in the range of 0.05-1.5 nm, manufactured by BIA separations and used for bioaffinity related separation applications, UNO is a polyacrylate based monolith marketed by Bio-Rad, Chromolith is modified silica based monolith marketed by Merk industries having pore size 1-2 nm, other modified cellulose and polymethacrylate based monoliths are sepragen, Isco marketed by seprasorb and SWIFT respectively. Industrially marketed monoliths are mainly used for purification of biomolecules like IgG, DNA plasmids and virus particles. In

comparison with porous particle media monoliths DNA binding capacity is about 50 times higher whereas viruses binding capacity is 100 times higher.

1.5.8 High pressure tangential flow filtration (HPTFF) coupled with cation exchanger

HPTFF is a membrane based technique used for downstream purification of biomolecules such as proteins, enzymes, endotoxins and monoclonal antibodies, it also clear viruses with removal of degradation products. In comparison to other separation techniques major advantages of this technique are simultaneous purification, concentration and exchange of components used for elution responsible for combination of several separation steps in individual unit operation. This process in integration with cation exchanger provide hybrid process which is best alternative for affinity chromatographic methods in terms of purity as well as cost of final product. In this integrated hybrid process pH and ionic strengths can easily be controlled and adjusted to have advantage of electrostatic interactions that involve between crude mixture to be purified and membrane. The other advantage of coupling these two techniques is that it involve separation of different charged particles eventhough they have similar size. Incorporation of cation exchangers helps to improve selectivity as well as purity of product with higher efficiency. Ebersold et. al. demonstrates utilisation of technique for separation of biomolecules based on variation in charge which will enhance separation several times.

1.6 Conclusion:

Hybrid and integrated processes plays important role in production of biopharmaceutical products with improved yield as well as purity at lower cost. As upstream process development work for biopharmaceuticals especially for monoclonal antibodies; enzymes and proteins achieved maximum success in terms of yield as well as productivity the downstream processing still trapped in expensive chromatographic techniques. In order to make downstream process robust and cost effective hybrid process technologies found to have revolutionary potential. As a individual unit operation aqueous two phase extraction have competitive advantages in terms of host protein removal and product recovery but in terms of purity it has some limitations which will be easily overcome by integrated hybrid technologies. Competitiveness of various integrated technologies were illustrated in following table, which shows that magnetic ATPS with boronic acid MPs found to be more effective. Hybrid monoliths having ligand-22/8 coated magnetic MPs are also effective but in terms of higher processing capabilities magnetic ATPS were more beneficial.

Table 1.6: Comparison of the product purity and yield in various hybrid processes described

Technology Type	Biomolecule type	System Used	Particles Used	Purity	Yield	References
HGMS integrated with ATPS	human IgG	PEG/Dextran	MNP-Boronic Acid	98	92	[29]
Magnetic particles integrated with micellar ATPS	Lysozyme	Triton – X	MNP-PVA	74	80	[13]
Affinity precipitation integrated with ATPS	Chitinases	PEG-Phosphate	--------	58	86	[121]
Monoliths incorporated with magnetic particles	human IgG	Chitosan monolith	MNP-22/8	96	95	[12]
High pressure tangential flow coupled with cation exchanger	rhuMAb	--------	-------	95	75	[31]
Traditional HGMS	human IgG	--------	MNP-Boronic Acid	67	74	[28]
Traditional ATPS	human IgG	PEG/ Citrate	--------	65	99	[7]
Temperature sensitive ATPS	human IgG	UCON/dextran/ TEG-COOH	--------	76	85	[32]
ATPS incorporated with chemically modified polymer and free ligand	human IgG	PEG-(COOH)2/ Dextran	--------	82	96	[8]

Reference:

1. Adriaenssens E.M., L. S. M., Vandersteegen K., (2012). CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. Virology, 434(2), 265-270.
2. Aguilar O. et. al. (2006). Direct comparison between ion-exchange chromatography and aqueous two-phase processes for the partial purification of penicillin acylase produced by E. coli. J. Chromatogr. B, 835, 77-83.
3. Aguilar O. et. al. (2010). Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soyabean proteins. Sep. Sci. Technol., 45, 2210-2225.

Ahmad A. L., D. C. J. C., Zulkali M. M. D., (2008). Sep. Purif. Technol., 62, 704-710.

4. An X., S. Z., Zeng H.,. (2003). Preparation of highly magnetic chitosan particles and their use for affinity purification of enzymes. Journal of Chemical Technology & Biotechnology, 78(5), 596-600. Antov M. and Omorjan R. (2009). Bioprocess Biosyst, 32, 235-240
5. Asenjo J.A. and Andrews B.A. (2011). Aqueous two-phase systems for protein separation: a perspective. J. Chromatogr. A, 1218(49), 8826-8835.
6. Asenjo JA, A. B. (2009). Protein purification using chromatography: selection of type, modelling and optimization of operating conditions. J. Mol. Recognit., 22(2), 65-76
7. Azevedo A.M. et al. (2009). Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep. Purif. Technol., 65, 31-39.
8. Azevedo A.M. et. al., & (2009). Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography. J. Chromatogr. B, 877(50 - 58).
9. Azevedo A.M., R. P. A. J., Ferreira I.F., Aires-Barros M.R.,(2008). Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction and size-exclusion chromatography. J. Chromatogr. A, 1213, 154-161.
10. Azevedo A.M.et.al. (2009). Chromatography-free recovery of biopharmaceuticals through aqueous two-phase processing. Trends in Biotechnol, 27(4), 240-247.
11. Bandeira V., P. C., Rodrigues A.F., (2012). Downstream processing of lentiviral vectors: releasing bottlenecks. Hum. Gene Ther. Method, 23(4), 255-263.
12. Barbosa H.S.C., H. A. V., Brocchini S., Slater N.K.H., Marcos J.C. (2010). Dual affinity method for plasmid DNA purification in aqueous two-phase systems. J. Chromatogr. A, 1217(9), 1429-1436.
13. Becker, J. S., O.R.T. Thomas, and M. Franzreb. (2009). Protein separation with magnetic adsorbents in micellar aqueous two-phase systems. Sep. Purif. Technol., 65, 46-53.
14. Bensch M., S. B., Hubbuch J., (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.
15. Bensch M. et. al. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62, 2011-2021.
16. Bensch M., S. B., Hubbuch J,. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.
17. Bhambure R. et. al. (2011). High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol., 29, 127-135.
18. Biao N., J. W., Xuejun C.(2009). Preparation and recycling of aqueous two-phase systems with pH-sensitive amphiphilic termopolymer PADB. Biotechnol. Prog., 25(3), 820-824.

19. Bolognese B., N. B., Pico G.,(2005). Application of the aqueous two-phase systems of ethylene and propylene oxide copolymer-maltodextrin for protein purification. J. Chromatogr B, 814, 347-353.
20. Bolton G.R. et. al. (2011). Addressing the challenges in downstream processing today and tomorrow. Biopharm Int., 24, S8-S15.
21. C.O. Rangel - Yagui, A. P. J., D. Blankschtein. (2004). Two - Phase aqueous micellar systems - an alternative method for protein purification. Braz. J. Chem. Eng., 21(4), 531 - 544.
22. Chavez-Santoscoy A., B. J., Vermaas W., Ri-to-Palomares M.,. (2010). Chem. Eng. Technol., 33, 177-182
23. Chen J, T. J., Zhang Y, Wasserman A, Conley G, DiLeo M, Haimes E, Nixon AE, Ley A. (2010). The distinctive separation attributes of mixed-mode resins and their applications in monoclonal antibody downstream purification process. J Chromatogr A, 1217, 216-224
24. Chen S. et. al. (2009). A screening paradigm for the design of improved polymer-coated superparamagnetic iron oxide nanoparticles. . Journal of Materials Chemistry, 19(35).
25. Chusainow J. (2009). A study of monoclonal antibody producing CHO cell lines - what makes a stable high producer? Biotechnol. Bioeng.,102, 1182-1196.
26. Cramer S.M, a. H. M. A. (2011). Downstream bio processing: recent advances and future promise. Curr. Opin. Chem. Eng, 1, 27-37.
27. Dhadge V.L., H. A., Azevedo A.M., Aires-Barros MR., Roque C.A.,. (2013). Boronic acid modified magnetic particles for antibody purification. Royal society interface.
28. Dhadge V.L., P. M., Maria Reis, Azevedo A.M., Aires-Barros MR., Roque C.A.,(2014). Green polymer coated magnetic particles at the interface of biopurification. Royal society interface, Under review.
29. Dhadge V.L., A. A. M., Aires-Barros MR., Roque C.A., (2014). Magnetic aqueous two phase fishing A hybrid process technology for antibody purification. J. Chromatogr A.
30. Ditsch A. et. al. (2005). High-Gradient Magnetic Separation of Magnetic Nanoclusters. Industrial & Engineering Chemistry Research, 44(17), 6824-6836.
31. Faria J. T., S. F. C., Converti A.,Passos F. M. L.,Minim V. P. R.,Minim L. A., . (2009). J. Chromatogr., B 877, 3031-3037
32. Ferreira I.F. et al. (2008). Purification of human immunoglobulin G by thermoseparating aqueous two-phase systems. J. Chromatogr. A, 1195, 94-100.
33. Frakas T., S. H., Tjerneld F.,. (1996a). Partitioning of bmannanase and a-galactosidase from Aspergillus niger in Ucon/Reppal aqueous two phase system. Bioseparation, 6, 147-157.
34. Freitas S. et. al. (2009). Alternatives for the intermediate recovery of plasmid DNA: performance, economic viability and environmental impact. Biotechnol. J., 4, 265-278.

35. Gagnon P. (2008). The emerging generation of chromatography tools for virus purification. BioProcess Int, 6(S6), 24-30.

36. Gagnon P. (2009). IgG aggregate removal by charged hydrophobic mixed-mode chromatography. Curr. Pharm. Biotech., 10, 434-439.

37. Gagnon P. et. al. (2012). Technology trends in antibody purification. J. Chromatogr. A, 1221, 57-70.

38. Gao J. et. al. (2012). Antibody affinity purification using metallic nickel particles. Journal of Chromatography B, 895-896, 89-93.

39. Gerster P., K. E. M., Hammerschmidt N.,. (2013). Purification of infective baculoviruses by monoliths. J. Chromatogr. A, 1290, 36-45.

40. Goloverda G. et. al. (2009). Synthesis of ultrasmall magnetic iron oxide nanoparticles and study of their colloid and surface chemistry. . Journal of Magnetism and Magnetic Materials, 321(10), 1372-1376.

41. Gottschalk U. (2006). The renaissance of protein purification. Biopharm. Int., 19, 8-9.

42. Gottschalk U. (2012). The need for innovation in biomanufacturing. Nat. Biotechnol, 30, 489-492.

43. Guan Y., L. T. H., Treffry T.E., Zhou C.L.,Wilkinson P.B., . (1996). Enzyme Microb. Technol., 19(6), 446.

44. Guoqiang D., K. R., Mattiasson B.,. (1994). Integration of aqueous two-phase extraction and affinity precipitation for the purification of lactate dehydrogenase. J Chromatogr A, 668, 145-152.

45. Heebol-Nielsen A., J. S., Hobley TJ.,Thomas ORT.,(2004). Superparamagnetic cation-exchange adsorbents for bioproducts recovery from crude process liquors by high gradient magnetic fishing. Sep Sci Technol, 39, 2891-2914.

46. Heeboll-Nielsen A., D. M., Hubbuch JJ., Thomas ORT.,(2004). Superparamagnetic adsorbents for high gradient magnetic fishing of lectins out of legume extracts. Biotechnol Bioenng, 87, 311-323.

47. Holschuh K. and Schwammle A. (2005). Preparative purification of antibodies with protein A – an alternative to conventional chromatography. J Magn Magn Mater, 293, 345-348.

48. Horák D. et. al. (2007). Preparation and properties of magnetic nano- and microsized particles for biological and environmental separations. Journal of Separation Science, 30(11), 1751-1772.

49. Hubbuch J.J. and Thomas O.R.T. (2002). High gradient affinity separation of trypsin from porcine pancreatin. Biotechnol Bioeng, 79, 301-313.

50. Jain E, a. K. A. (2008). Upstream processes in antibody production - evaluation of critical parameters. Biotechnol. Adv., 26, 46-72.

51. Jefferis R. (2009). Glycosylation as a strategy to improve antibodybased therapeutics. Nature, 8, 226-234.

52. Jiang C. et. al. (2009). A mechanistic study of Protein A chromatography resin lifetime. J. Chromatogr. A, 1216, 5849-5855.
53. Jiang N. Q. and Zhao F. S. (1999). Ind. Microbiol, 29, 5-9
54. Jin M. et. al. (2010). Profiling of host cell proteins by two dimensional difference gel electrophoresis (2D-DIGE) - implications for downstream process development. Biotechnol. Bioeng., 105, 306-316.
55. Jonsson M. and Johansson H.O. (2003). Protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer. J Chromatogr A 983, 133-144.
56. Jungbauer A. and Hahn R. (2008). Polymethacrylate monoliths for preparative and industrial separation of biomolecular assemblies. J. Chromatogr. A, 1184(1-2), 62-79.
57. Jungbauer A. and Peng J. (2011). Continous bioprocessing: an interview with Konstantin Konstantinov from Genzyme. Biotechnology J.,6(12), 1431-1433.
58. Jyh P. C. and Fong L. J. (1995). Purification of trypsin by affinity precipitation and combining with aqueous two phase extraction Biotechnology techniques, 9(7), 461-466.
59. Kandula S. et. al. (2009). Design of a filter train for precipitate removal in mAb downstream processing. Biotechnol. Appl. Biochem., 54, 149- 155.
60. Kang S. et. al. (2009). Bioconjugation of poly (ethylene glycol) methacrylate)-coated iron oxide magnetic nanoparticles for magnetic capture of target proteins. Macromolecular Research, 17(4), 259-264.
61. Kepka C., C. E., Persson J., Stahl A., Lagerstedt T., Tjerneld F., Veide A., (2003). Extraction of an intracellular recombinant cutinase from E. Coli cell homogenate using thermoseparating aqueous two phase system. Journal of Biotechnology, 103 165-181.
62. Kramberger P., H. R. C., Herman R.E., Smrekar F., Peterka M.(2010). Purification of the Staphylococcus aureus bacteriophages VDX-10 on methacrylate monoliths. J. Virol. Methods, 166(1-2), 60-64.
63. Kuczewski M. et. al. (2011). A single-use purification process for the production of a monoclonal antibody produced in a PER.C6 human cell line. Biotechnology Journal, 6(1), 56-65.
64. Kulkarni N., V. A., Rao M.,(1999). Biochem. Biophys. Res. Commun., 225, 274-278
65. Kumar A., W. P. O., Kepka C., Galaev I.Yu, Mattiasson B., (2003). Purification of histidine-tagged single chain Fvantibody fragments by metal chelate affinity precipitation using thermo-responsive copolymers. Biotechnol Bioeng, 84, 495-503.
66. Lacki K.M. (2012). High-throughput process development of chromatography steps: advantages and limitations of different formats used. Biotechnol. J., 7, 1192-1202.
67. Lam H., K. M., Haynes C.A., D.I.C. Wang., D. Blankschtein.,. (2005). Affinity - enhanced protein partitioning in decyl D-glucopyranoside two-phase micellar systems. Biotechnol. Bioeng., 89(4), 381-392.

68. Li C. and Ouyang F. (2000). Biotechnol. Lett., 22, 843-847
69. Li M. et.al., K. J. W., Peeples T.L.,. (2002). Amylase partitioning and extractive bioconversion of starch using thermoseparating aqueous two-phase systems. J Biotechnol, 93, 15-26.
70. Liu HF., M. J., Winter C., (2010). Recovery and purification process development for monoclonal antibodies. mAbs, 2, 480-499.
71. Liu J. et. al. (2009). Comparison of camelid antibody ligand to Protein A for monoclonal antibody purification. Biopharm. Int. , 22, 35-43.
72. Liu, X., et al., 2004. Surface Modification and Characterization of Magnetic Polymer Nanospheres Prepared by Miniemulsion Polymerization. Langmuir, 20(23), 10278-10282.
73. Low D. et. al. (2007). Future of antibody purification. J. Chromatogr. B, 848, 48-63.
Lu J., L. D. Q., YaO S.J.,(2006). Preparation and application of novel EOPO-IDA-metal polymer as recyclable metal affinity ligand in aqueous two-phase system. Ind. Eng. Chem. Res., 45(5), 1774-1779.
74. Luechau F., L. T. C., Lyddiatt A.,(2011). Recovery of B19 virus-like particles by aqueous two-phase systems. Food Bioprod. Process, 89(4), 322-327.
75. Marcos J. C., F. L. P., Ramalho M. T., Cabral J. M. S., (2002). EnzymeMicrob. Technol., 31, 1006-1014
76. Mattiasson B., K. A., Ivanov A.E., Galaev I.Yu.,. (2007). Metalchelate affinity precipitation of proteins using responsive polymers. Nat Protoc, 2, 213-220.
77. Mazumder J., Z. J., Ray AK.,. (2010). Optimal design of liquid-solid circulating fluidized bed for continuous protein recovery. . Powder Technol. , 199(1), 32-47.
78. Meyer A., H. D., Gomes CSG, Hobley TJ, Thomas ORT, Franzreb M,. (2005). Demonstration of a strategy for product purification by high gradient magnetic fishing. Biotechnol Prog, 21, 244-254.
79. Muller-Spath T. et. al. (2008). Chromatographic separation of three monoclonal antibody variants using multicolumn countercurrent solvent gradient purification. Biotechnol. Bioeng., 100, 1166-1177.
80. Muller-Spath T, A. L., Strohlein G, Kornmann H, Valax P, Delegrange L, Charbaut E, Baer G, Lamproye A, Johnck M. (2010). Two step capture and purification of IgG2 using multicolumn countercurrent solvent gradient purification (MCSGP). Biotechnol Bioeng(107), 974-984.
81. Müller-Späth T, K. M., Aumann L, Ströhlein G, Morbidelli M. (2010). Increasing the activity of monoclonal antibody therapeutics by continuous chromatography Biotechnol. Bioeng, 107(4), 652-662.
82. Naganagouda K. and Mulimani V.H. (2008). Aqueous two-phase extraction (ATPE): an attractive and economically viable technology for downstream processing of Aspergillus oryzae a-galactosidase. Process Biochem, 43, 1293-1299.

83. Nfor B.K. et. al. (2012). Model-based high-throughput process development for chromatographic whey protein separation. Biotechnol. J. , 7, 1221-1232.
84. Nfor B.K. et. al. (2013). Model-based rational methodology for protein purification process synthesis. Chem. Eng.Sci., 89, 185-195.
85. Nfor B.K., V. P. D. E. M., Hubbuch J., Ottens M.,. (2009). Rational and systematic purification process development: the next generation. Trends Biotechnol, 27, 673-679.
86. Nishio K. et. al. (2008a). Development of novel magnetic nano-carriers for high-performance affinity purification. Colloids and Surfaces B: Biointerfaces, 64(2), 162-169.
87. Oelmeier S.A., D. F., Hubbuch J.,. (2011). Application of an aqueous two-phase systems high-throughput screening method to evaluate mAb HCP separation. Biotechnol. Bioeng., 108(1), 69-81.
88. Öztürk, N., et al.,2007.Silane-Modified Magnetic Beads: Application to Immunoglobulin G Separation Biotechnology Progress, 23(5), 1149-1156.
89. Pereira M., W. Y. T., Venaˆ ncio A., Teixeira J.,. (2003). Aqueous twophase extraction using thermoseparating polymer: a new system for the separation of endo-polygalacturonase. Biochem Eng J, 15, 131-138.
90. Pericin D. M., P. S. Z. M., Popovic L. M. R.,. (2009). Biotechnol. Lett., 31, 43-47
91. Persson J., A. D. C., Lester P.M., . (2005). Biotechnol. Bioeng., 90(4), 442.
92. Persson J. et.al. (2000). Aqueous polymer two-phase systems formed by new thermoseparating polymers. Bioseparation, 9, 105-116.
93. Platis D. and Labrou N.E. (2006). Development of an aqueous two phase partitioning system for fractionating therapeutic proteins from tobacco extract. J. Chromatogr. A, 1128, 114-124.
94. Platis D., D. J., Fischerc R., Ma J. K. C., Labrou N. E., (2008). J. Chromatogr., A, 1211, 80-89
95. Poechlauer P., M. J., Broxterman R, Gregertsen B, Ridemark M. . (2012). Continuous processing in the manufacture of active pharmaceutical ingredients and finished dosage forms: an industry perspective. Org. Proc. Res. Devel., 16(10), 1586-1590.
96. Porto C. S., P. T. S., Nascimento K. S., Teixeira E. H., Cavada B. S., Filho J. L. L., Porto A. L. F., . (2011). Biochem. Eng. J., 53, 165-171
97. PortoT. S., S. G. M. M., Porto C. S., Cavalcanti M. T. H.,Neto B.B., Lima- Filho J. L., Converti A., Porto A. L. F., Pessoa A.,(2008). Chem. Eng. Process., 47, 716-721
98. Qian H. et al. (2009). The efficient and specific isolation of the antibodies from human serum by thiophilic paramagnetic polymer nanospheres. Biotechnology Progress, 25(2), 376-383.
99. Qian H.et. al. (2010). Using thiophilic magnetic beads in purification of antibodies from human serum. Colloids and Surfaces B: Biointerfaces, 75(1), 342-348.
100. Rahimpour F., M. G., Feyzi F.,Maghsoudi S.,Hatti Kaul R.,. (2007). J. Chromatogr A, 1141, 32-40

101. Rahman M.M. et. al. (2011). Highly temperature responsive core-shell magnetic particles: Synthesis, characterization and colloidal properties. Journal of Colloid and Interface Science, 360(2), 556-564.
102. Reichert J, a. V.-A. V. (2007). Development trends for monoclonal antibody cancer therapeutics. Nat. Rev. Drug Discov. , 6, 349-356.
103. Rito-Palomares M., N. A., Miranda L.,Flores C.,Galindo E.,Carreon L. S.,(2001). Enzyme Microb. Technol. , 28, 625-631
104. Rito-Palomares M. (2004). Practical application of aqueous two-phase partition to process development for the recovery of biological products. J. Chromatogr. B, 807, 3-11.
105. Rogers M. et. al. (2009). Development of a rapid sanitization solution for silica-based protein A affinity adsorbents. J.Chromatogr. A 1216, 4589-4596.
106. Roobol - Boza M., D. V., Doverskog M., Barrefelt A., Lindqvist F., Oppermann K., Tjerneld F.,. (2004). Membrane protein isolation by in situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems. J. Chromatogr. A, 1043, 217 - 223.
107. Rosa P.A.J., A. A., Sommerfeld S, Mutter M, Baecker W, Aires-Barros MR. . (2013). Continuous purification of antibodies from cell culture supernatant with aqueous two-phase systems: from concept to process. Biotechnol. J., 8(3), 352-362.
108. Rosa P. A. J., A. A. M., Aires-Barros M. R., . (2007). J. Chromatogr A, A1141, 50-60.
109. Rosa PAJ., A. A., Sommerfeld S, Baecker W, Aires-Barros MR.,. (2011). Aqueous two-phase extraction as a platform in the biomanufacturing industry: economical and environmental sustainability. Biotechnol. Adv., 29(6), 559-567.
110. Ruuls S. (2008). Novel human antibody therapeutics: the age of the Umabs. Biotechnol. J., 3, 1157-1171.
111. Sanaie N. et. al. (2012). Applying high-throughput methods to develop a purification process for a highly glycosylated protein. Biotechnol. J., 7, 1241-1251.
112. Sandoval G. et. al. (2012). Elution relationships to model affinity chromatography using a general rate model. J. Mol. Recognit, 25, 571-579.
113. Shukla AA. (2007). Downstream processing of monoclonal antibodies - application of platform approaches. J. Chromatogr. B, 848, 28-39.
114. Shukla A and Kandula J. (2008). Harvest and recovery of monoclonal antibodies from large-scale mammalian cell culture. Biopharm. Int., 7(34-45).
115. Shukla A. et. al. (2007). Protein aggregation kinetics during Protein A chromatography - case study for an Fc fusion protein. J. Chromatogr. A, 1171, 22-28.
116. Shukla A. et. al. (2008). Demonstration of robust host cell protein clearance in biopharmaceutical downstream processes. . Biotechnol. Progr., 24, 615-622.

117. Shukla A.A, a. T. J. (2010). Recent advances in large scale production of monoclonal antibodies and related proteins. Trends Biotechnol, 28, 253-261.
118. Smrekar F., C. M., Štrancar A., Podgornik A., . (2011). Characterisation of methacrylate monoliths for bacteriophage purification. J. Chromatogr. A, 1218(17), 2438-2444.
119. Smrekar F., P. A., Ciringer M.,. (2010). Preparation of pharmaceutical-grade plasmid DNA using methacrylate monolithic columns. Vaccine, 28(8), 2039-2045.
120. Srinivas N.D., N. A. V., Raghavarao K.S.M.S., . (2002). Process Biochem., 38, 387.
121. Teotia S., L. R., Gupta M.N., . (2004). Chitosan as a macroaffinity ligand. Purfication of chitinases by affinity precipitation and aqueous two-phase extractions. J Chromatogr A, 1052, 85-91.
122. Thommes J. and Etzel M. (2007). Alternatives to chromatographic separations. Biotechnol. Progr., 23, 42-45.
123. Tugcu N. et. al. (2008). Maximizing productivity of chromatographic steps for purification of monoclonal antibodies. . Biotechnol. Bioeng., 99, 599-613.
124. Van Wijnendaele F., G. D., Simonet G., . (1987). US Patent 4,683,294,.
125. Vogel J.H. et. al. (2012). A new large-scale manufacturing platform for complex biopharmaceuti. Biotechnol. Bioeng. , 109, 3049-3058.
126. Voitl A, M. S. T., Morbidelli M. (2010). Application of mixed mode resins for the purification of antibodies. J. Chromatogr A, 1217, 5753-5760.
127. Walsh G. (2010). Biopharmaceutical benchmarks Nat. Biotechnol. , 28, 917-924.
128. Wang W., W. J. F., Ning B., Xia J.N., Cao X.J.,. (2008). Preparation of a novel light-sensitive copolymer and its application in recycling aqueous two-phase systems. J. Chromatogr. A, 1205(1-2), 171-176.
129. Xu H. et. al. (2011). Antibody conjugated magnetic iron oxide nanoparticles for cancer cell separation in fresh whole blood. Biomaterials, 32(36), 9758-9765.
130. Ye J. (2009). High-level protein expression in scaleable CHO transient transfection. Biotechnol. Bioeng., 103, 542-551.
131. Ying X. U., G.-q. H. E., Jing-jun L. I., Zhejiang J.,. (2005). Univ. SCI, 6B, 1087-1094.
132. Zandian M. and Jungbauer A. (2009). Engineering properties of a camelid antibody affinity sorbent for immunoglobulin G purification. J. Chromatogr. A, 1216, 5548-5556.
133. Zhao G. et. al. (2009). Ligands for mixed-mode protein chromatography: principles, characteristic and design. J. Biotechnol.(144), 3-11.
134. Zheng W., G. F., Gu H., . (2005). Magnetic polymer nanospheres with high and uniform magnetite content. Journal of Magnetism and Magnetic Materials, 288, 403-410.
135. Zhi W., S. J., Ouyang F., Bi J., . (2005). Biotechnol. J.(118), 157-165.

CHAPTER 2

BORONIC ACID MODIFIED MAGNETIC MATERIALS FOR ANTIBODY PURIFICATION

SUMMARY

Aminophenyl boronic acids can form reversible covalent ester interactions with cis-diol containing molecules, serving as a selective tool for binding glycoproteins as antibody molecules which possess oligosaccharides in both the Fv and Fc regions. In this study, amino phenyl boronic acid (APBA) magnetic particles (MPs) were applied for the magnetic separation of antibody molecules. Iron oxide magnetic particles were firstly coated with dextran to avoid non-specific binding and then with 3-glycidyloxypropyl trimethoxysilane (GLYMO) to allow further covalent coupling of APBA (APBA_MP). When contacted with pure protein solutions of human IgG (hIgG) and bovine serum albumin (BSA), APBA_MP bound 170 ± 10 mg hIgG/g MP and eluted 160 ± 5 mg hIgG/g MP, while binding only 15 ± 5 mg BSA/g MP. The affinity constant for the interaction between hIgG and APBA_MP was estimated as 4.9×10^5 M^{-1} (Ka) with a theoretical maximum capacity of 492 mg hIgG adsorbed/g MP (Qmax), whereas control particles bound a negligible amount of hIgG and presented an estimated theoretical maximum capacity of 3.1 mg hIgG adsorbed/g MP (Qmax). APBA_MPs were also tested for antibody purification directly from CHO cell supernatants. The particles were able to bind 98% of IgG loaded and to recover 95% of pure IgG (purity greater than 98%) at extremely mild conditions.

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2.1 Introduction

Antibodies and antibody derived molecules represent an important group of biopharmaceuticals, to which several improvements have been reached at the upstream level over the last years [1]. Developments have also been made at the side of downstream processes with several new non-chromatographic techniques gaining increased relevance, namely aqueous two phase systems [2], crystallisation/precipitation [3], and magnetic fishing [4]. Iron oxide magnetic particles (MPs) find applications as nanomedical vectors [5], carriers for biocatalysis, biomedical applications[6], biosensing purposes [7], magnetofecation agents [8], as well as matrices for purification processes [9]. The great interest in these materials, in particular for bioseparation purposes, arises from properties such as superparamagnetism, high surface to volume ratio and fast binding kinetics, as well as an extreme versatility for chemical derivatization [10]. MPs show superior properties for modification utilising artificial as well as natural ligand. The particle effectiveness for application from biopharmaceutical as well as from all other application point of view relies on superparamagnetism property. Superparamagnetism is the lack of magnetic memory, a property ideally suited for reversible adsorption-desorption processes as the ones employed in protein purification. Using this property for crude biological mixture purification makes process much more easier. The technique based on this property initially make suitable and preferential contact between molecule of interest from mixture after that MPs were collected using external magnetic field. The particles collected were eluted using buffer which breaks bond between attached molecule and magnetic particle modified ligand. Commonly MPs consist of stable colloidal MPs embedded in a polymer or of polymer-coated iron-oxide crystal clusters. The surface to volume ratio is intrinsically correlated with the final hydrodynamic size of the MPs or clusters [11], which can be controlled by tuning magnetic particle synthesis and modification [12]. In order to render MPs active towards binding specific biomolecules, polymer-coated particles can be chemically modified with suitable functional groups like oleylamine [13], and then with ligand similarly to what is done in chromatographic resins [14]. When considering the purification of antibody molecules, magnetic fishing is particularly suited for an initial capture step, usually conducted with protein-A functionalised chromatographic resins [15]. There are protein A-coated MPs commercially available for the purification of antibodies, and previous studies indicated that other types of functionalised MPs can be also suitable for the capture step. Protein A coated MPs that are available in the market are expensive which limits the use of MPs for large scale applications. Protein A is a biological protein recovered from staphylococcus aureus. The process of synthesis and recovery of protein A is costlier which is responsible for overall higher cost of pure protein fraction.To have effective particles at afforded prices alternative to this biological ligand is must which can be easily interchanged using chemical synthesis based ligands. In particular, our groups have shown that custom made MPs coated with small and robust synthetic ligands mimicking protein A [16], and commercial boronic acid coated MPs [17] represent interesting and viable options for capturing antibodies in purification processes. Commercial boronic acid MPs allowed the direct and selective capture of a

human mAb from a CHO feedstock, where binding and elution conditions were tuned to reach maximum yield and final purity [17]. However, the high cost of these commercial particles turns unviable the scale-up of the purification process. Therefore, encouraged by the results obtained with commercial boronic acid particles, this work focused on the development of iron oxide MPs coated with dextran, a polymer already shown to greatly reduce non-specific interactions [16], further chemically modified with aminophenyl boronic acid by three distinct routes, which resulted in the development of an easy and scalable procedure for MP synthesis and subsequent application on the one-step recovery of antibodies.

2.2. Materials and methods

2.2.1. Materials

The reagents employed were of highest available purity and used without further purification. 3-glycidyloxypropyl trimethoxysilane (GLYMO), ferric sulfatehydrate [$Fe_2(SO_4)_3$].H_2O, ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), (3-aminopropyl) triethoxysilane (APTES), sodium silicate ($Na_2SiO_3.5H_2O$), amino phenyl boronic acid (APBA), tris(hydroxymethyl)amino methane, dextran from *Leuconostoc mesenteroides*, glutaraldehyde, tetraethoxy silane (TEOS), gum arabic, anthrone, sodium hydroxide, and alizarin red were purchased from Sigma – Aldrich. Glycine was purchased from Acros. Ninhydrin and ammonium hydroxide were purchased from Fluka. Bichinchoninic acid (BCA) kit from Sigma was used for protein quantification assay. The reagents used for SDS-PAGE gels were, ammonium persulphate (APS), N,N,N,N–Tetramethylethylenediamine (TEMED), 30% acrylamide/bisacrylamide-solution, sodium dodecyl sulphate micropelletes, silver stain plus kit purchased from BIO-RAD; The proteins employed were human immunoglobulin from Octapharma (95%) and albumin from bovine serum (98%) purchased from Sigma-Aldrich. All the spectrophotometric and spectrofluorometric measurements were taken with the microplate reader – Tecan infinite f 200 from Tecan. The mini-protean tetra system from BIO-RAD was utilized for the electrophoresis of SDS-PAGE gels.

2.2.2. Synthesis and characterization of magnetic particles

2.2.2.1. Synthesis of iron oxide magnetic particles

The synthesis of iron oxide magnetic particles followed the co-precipitation method, using ferric and ferrous sulphate. For synthesis 2.36 g $FeSO_4.7H_2O$ and 6.79 g $Fe_2(SO_4)_3.5H_2O$ were dissolved in 150 mL deionised water, preheated to 80^0C in an inert atmosphere. When the temperature reached 80^0C, 50 ml ammonium hydroxide of 25 % (v/v) concentration was added and vigorously stirred for 30 minutes at 1200 rpm. After 30 minutes the resulting magnetic particles were collected using a permenant magnet and thoroughly washed with deionized water.

2.2.2.2. Silica and TEOS coating of iron-oxide magnetic particles

The silica coating was carried out according to method described by Zi-An Lin [18] with minor modifications. Briefly, 120 ml aq. solution containing 3.4 g $Na_2SiO_3.5H_2O$ and 0.4 g NaOH was introduced into the resultant particles after removal of deionized water from them using permenant magnet. Before addition the mixture was sonicated for 5 min. to obtain homogenous solution of sodium silicate. The

coating process was carried out using mechanical stirring for 30 minutes at slow agitation which will only allow the gentle mixing of solution and particles. This step was followed by the dropwise addition of 2 ml of 1M HCl. Further, the particles mixture was added with 200 µL TEOS dissolved in 1 mL ethanol (use excess TEOS in order to have better coating), addition was carried out with gentle mechanical stirring for 1 h. The entire process was carried out at room temperature. At the end of coating process the particles so prepared were washed with deionized water.

The magnetic particles so prepared were then diluted to a concentration of 10 mg/ml and then coated with dextran. Aqueous solutions of dextran (2 and 4 g dextran separately dissolved in 50 ml distilled water) were prepared and added to 50 ml magnetic particles to yield MP-DX-1 and MP-DX-2, respectively. The mixtures were stirred mechanically for 2 h at room temperature, after which particles were washed with water. The amount of dextran released during the washings was quantified by the anthrone method, followed the procedure described in [16].

2.2.2.3. APBA coating of dextran coated magnetic particles

Three different methods were tested for the modification of MP-DX-2 with amino phenyl boronic acid (APBA). A first method consisted of redispersion of 50 ml MP-DX-2 (10 mg/ml MP) in 50 ml ethanol by ultrasonication, followed by addition of 1 ml APTES and stirred mechanically for 1 h at room temperature. The resultant particles were washed with distilled water and then re-dispersed in 25 ml of PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) containing 10 ml 25 % glutaraldehyde, and left under mechanical stirring for 30 minutes. After that, particles were washed several times with distilled water and then dispersed in 50 ml PBS solution containing 0.5 g APBA (previously neutralised with base) and 0.2 g sodium cyanoborohydried. The mixture was then stirred mechanically for 3 h at room temperature. The obtained particles (MP-BA-1) were washed five times with distilled water and used for purification study.

A second method involved the reaction of MP-DX-2 with ammonium persulfate (APS) to form a complex organic net to hold the APBA molecule on the surface of MP yielding MP-BA-2. Briefly, 50 ml of MP-DX-2 (10 mg/ml) reacted with 4 g of APS and 0.4 g of neutralised APBA, for 2h at room temperature with mechanical stirring, after which particles were washed with distilled water.

A third method included the reaction of MP-DX-2 with GLYMO to introduce active epoxide groups for further reaction with the free amine of APBA. 50 ml of MP-DX-2 having concentration of 10 mg/ml were reacted with GLYMO for 3 h, at room temperature under mechanical stirring, after which 0.4 g of APBA (previously neutralised with NaOH) were added to the mixture and left reacting overnight. After completion of reaction the final MP-BA-3 were washed 5 times with distilled water and used for further study. The boronic acid particles synthesis process scheme is demonstrated in following figure – 2.1.

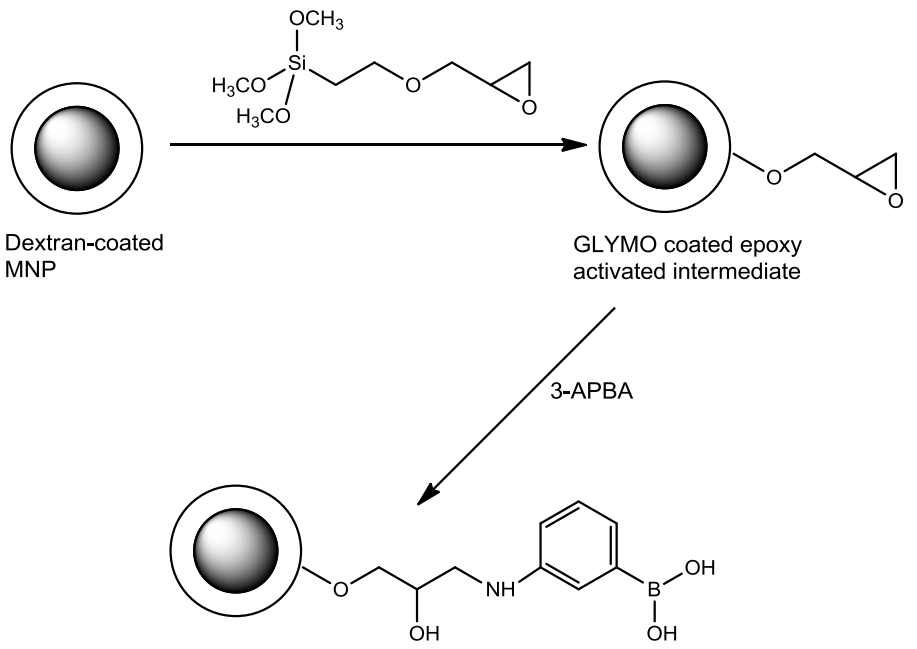


Figure: 2.1 A) Modification of dextran-coated MP *via* the GLYMO route. GLYMO coating leaves a reactive epoxide moiety which is subsequently reacted using 3-amino phenyl boronic acid (3-APB) to give the APBA-functionalised product. **B)** Alky or aryl boronic acids react with *cis*-diol hydroxyls on 6-membered pyranose and five membered furanose constituting carbohydrates under alkaline conditions to give boronate esters. Note the change from trigonal planar to tetrahedral geometry upon esterification, leaving a negative charge on the boron atom.

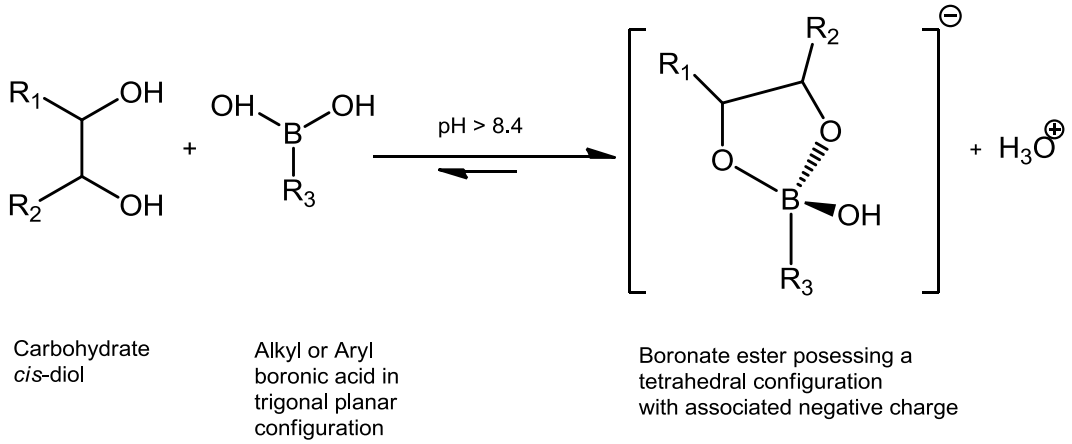


Figure: 2.2 B) Alky or aryl boronic acids react with *cis*-diol hydroxyls on 6-membered pyranose and five membered furanose constituting carbohydrates under alkaline conditions to give boronate esters. Note the change from trigonal planar to tetrahedral geometry upon esterification, leaving a negative charge on the boron atom.

The prepared MPs were characterized by using a vibrating sample magnetometer (DSM 880 VSM) at INESC-MN facilities (Lisbon, Portugal). The samples were prepared in milli-Q water with a concentration of 6.1 mg/ml and were used 30 µl of each sample in a vertical quartz rod. Transmission electron microscopy (TEM) was utilized for the characterization of particle morphology and estimation of the size of the magnetic core. The dried particle samples were prepared by evaporating dilute suspensions on a carbon-coated film and TEM performed in an analytical TEM Hitachi 8100 with rontec standard EDS detector and digital image acquisition. For all supports the physical properties (hydrodynamic diameters and zeta potential) were determined by dynamic light scattering (DLS), using a Zetasizer Nano ZS from malvern. For these analyses, samples with a final concentration of 0.05 mg/ml in milli-Q water were prepared.

2.2.2.3. Binding pure protein solutions to MPs

A 0.5 ml volume of MP-BA-3 with a concentration of 10 mg/ml was washed sequentially with 0.5 ml regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and UHP water and with elution buffer (1M Tris-HCl of pH 8.5) followed by 5 times washing with binding buffer (20 mM HEPES,pH-8.5). After that the supernatant was removed and resuspended in 0.5 ml protein sample (BSA or IgG of concentration 1 mg/ml in binding buffer) and incubated for 15 minutes at room temperature having agitation speed of 300 rpm. The supernatant was recovered and particles were washed five times with 0.5 ml of binding buffer, followed by elution with 0.5 ml (5 times) of elution buffer. All the washes were collected, centrifuged and separated in a magnetic support before being quantified by the BCA method.

2.3. Characterization of boronic acid coated particles

2.3.1 Binding of alizarin red to MPs

The alizarin red test was carried using a 5 mM stock solution of Alizarin red. A 250-µl sample of MPs (10 mg/ml) was incubated with the 250-µl alizarin red solution for 15 minutes. After incubation, particles were first washed two times with 250 µl binding buffer followed by five times washing using 250-µl elution buffer. The entire analysis was carried out using two types of buffer conditions i.e. phosphate-glycine and HEPES-Tris,HCl. In the case of phosphate-glycine, the adsorption and elution condition consisted of the phosphate buffer at pH-7.4 and glycine buffer of pH-11; while for HEPES-Tris,HCl consisted of HEPES buffer at pH-8.5 and Tris,HCl at pH-8.5. The major interest in using alizarin red test was to detect florescence to ascertain the presence of APBA coating on the surface of MPs.

2.3.2. Partition equilibrium Studies

Partition equilibrium experiments were performed with solutions of human IgG (0-25 mg/ml; 250-µl) in binding buffer (20 mM HEPES) and 250-µl at 10 mg/ml of MP-BA-3. For control MP-DX-2 were used in order to study relative adsorption. The samples were incubated for 12 h at room temperature, after which the supernatants were collected and the amount of free protein quantified by the BCA protein assay. Experimental data fitted with scatchad plot and was represented by,

$$q = \frac{Qmax.Ceq}{Kd + Ceq}$$

In which q is the amount of bound protein in equilibrium per volume of solid support, Ceq is the concentration of bound protein in equilibrium, corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent) and the dissociation constant.

2.3.3. Particle Reuse Study

After studying the supports for hIgG purification, the best supports MP-BA-3 were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The APBA functionalized (MP-BA-3) (500 µl with 10 mg/ml) were tested with 500 µl of each of the pure hIgG solution, by incubating for 15 minutes at room temperature. The liquid in which particles were suspended were removed by magnetic separation and then washed two times with 500 µl binding buffer (20 mM HEPES, pH_8.5). After washing, MPs were eluted five times using 500 µL elution buffer (1M Tris,HCl, pH_8.5). All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

2.3.4. Studies of incubation time

Time required for hIgG to adsorb on the surface of magnetic particles having functionalised APBA molecules on its surface was studied in order to finalise exact incubation time to be provided to have optimum separation ability. In this study five different test samples of MP-BA3 (10 mg/ml concentration) having 500 µl volume were incubated at room temperature with 500 µl hIgG solution (1 mg/ml) for different time intervals mainly 5,8,10,12 and 15 minutes respectively. Incubation was followed by washing the particles two times with 500 µl binding buffer (20 mM HEPES, pH 8.5) and five times elution using elution buffer (1M Tris-HCl of pH 8.5), in order to know maximum protein that we able to elute. All washes were collected and quantified by BCA method.

2.4 Purification of antibodies from unpurified mAb solutions

After studying the magnetic supports with pure solutions of hIgG, the selected magnetic material (MP-BA3) and the control particles (MP-DX2) were tested with CHO cell culture supernatants. 500 µl MP-DX-2 and MP-BA3 (10 mg/ml) were incubated with 500 µl of the crude extract respectively, without any pre-treatment, for 15 minutes at room temperature. Supernatants were collected and the particles were washed five times with 500 µl binding buffer (20 mM HEPES, pH 8.5). After washing, MPs were eluted using elution buffer (1M Tris-HCl of pH 8.5), in order to study the best elution conditions. All collected samples were quantified by affinity chromatography using Akta purifier system from GE healthcare (Uppsala, Sweden), using porous protein A affinity column from applied biosystems (Foster City, CA, USA). The BCA method and gel electrophoresis (12.5% acrylamide/bisacrylamide) in denaturing conditions by SDS-PAGE was also used for further analysis.The respective gels were prepared according to a standardized protocol. The low molecular weight marker and the samples for running gels were

prepared by adding 2.5 and 10 µl of each sample, respectively, and 5 µl of sample buffer and boiled for two minutes immediately before applying to the gel. The gel was run for 80 minutes at 150 V and 250 mA by adding an electrophoresis Tris – Glycine buffer (SDS-PAGE). For detection of the protein bands, the gel was stained with silver stain BIORAD kit.

2.5. Results and discussion

2.5.1. Selection of the magnetic materials for antibody adsorption

An ideal support for affinity based separations must be inert but still present chemical groups for further handling of specific receptors. The non-specific binding of bare iron oxide is a serious concern for selective adsorption and desorption in protein magnetic fishing. The crucial challenge of non-specific binding can be overcome by coating iron oxide particles with biopolymers. We have previously reported the employment of dextran as a preferential biopolymer to introduce an inert and functional layer onto MPs [16]. Dextran, when compared with other negatively charged biopolymers previously tested (e.g. gum Arabic and CM-dextran), presents an increased inertness probably due to its neutral nature and the avoidance of undesired electrostatic interactions [19].

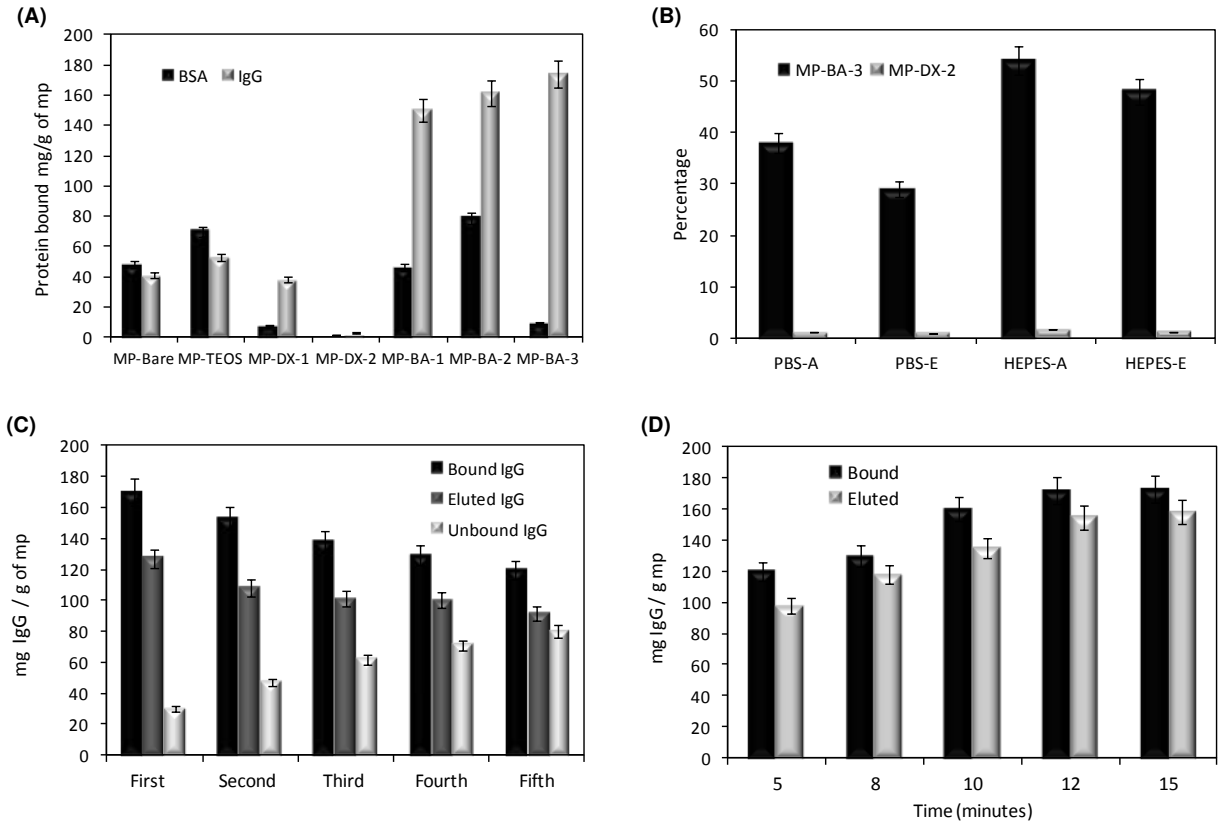


Figure: 2.2 (A) Quantity of BSA and IgG bound to the support under different modification stages. MP-Bare (Fe_3O_4 magnetic particles), MP-TEOS (tetraethoxy silane coated magnetic particles); MP-DX-1 (low

concentration dextran coated particles), MP-DX-2 (high concentration dextran coated particles), MP-BA1 (high concentration dextran coated mp modified using glutaraldehyde method), MP-BA2 (high concentration dextran coated mp modified using ammonium persulfate method), MP-BA3 (high concentration dextran coated mp modified using GLYMO method). **(B)** Alizarin red dye adsorption results on APBA mp modified with GLYMO (MP-BA-3) and MP-Bare which acts as control. PBS-A and PBS-E was quantity of dye adsorbed and eluted using phosphate – glycine buffer (pH 7.4 and 11). HEPES-A and HEPES-E was quantity of dye adsorbed and eluted using HEPES – Tris buffer (Both at pH 8.5). **(C)** Regeneration results for GLYMO modified APBA coated MP (MP-BA-3) in mg of protein bound per gm of mp. **(D)** Quantity of IgG bound to the mp support at various time periods.

Table: 2.1. Quantity of BSA and IgG bound and eluted from the support under different modification stages low concentration dextran coating is represented by DX-1 whereas high concentration dextran coating is represented by DX-2. (Binding buffer-20 mM HEPES of pH 8.5; Elution Buffer- 1 M Tris-HCl of pH 8.5). (n=4)

	IgG Bound(mg/g)	IgG Eluted(mg/g)	BSA Bound(mg/g)	BSA Eluted (mg/g)
MP-TEOS	53±4	12±3	70±6	23±5
MP-DX-1	38±5	14±4	8±2	3±3
MP-DX-2	3±4	1±4	2±4	2±1
MP-BA-3	173±2	166±3	10±2	4±2

In this work, the produced MPs were initially tested for binding to pure solutions of human IgG (hIgG, the target biomolecule) and bovine serum albumin (BSA, a model non-glycosylated contaminant protein) (Table 2.1 and Figure 2.2A). Bare MPs and TEOS coated MPs show non-specificity as they bound between 45-80 mg protein/g of magnetic support. To decrease this undesired reactivity, particles were coated with a low and a high concentration of dextran. It has been suggested that the dominant mechanism of the interaction between dextran and magnetic particles is the formation of collective hydrogen bonding between dextran hydroxyl groups and iron oxide particle surface. Coating of bare magnetic particles with silicon dioxide and tetraethoxysilane creates a spongy adsorptive surface with high adsorption capacity to polymeric substances. The silica coated structure is a network of silicon atoms connected by oxygen atoms and saturated with hydroxyl groups. The external coating of silica easily adsorb efficient amount of dextran solution on its surface. The maximum quantities of dextran coated on the surface of TEOS coated MPs were 1.1 (mg/g of MP) for low and 1.4 (mg/g of MP) for the higher concentration, respectively. It was observed that after biopolymer coating, non-specific binding of BSA was reduced to 8 mg/g of support, for the low concentration of dextran coating and almost negligible for the high concentration (Table 2.1 and Figure 2.2 A). The major reason behind a reduction in nonspecific binding is the creation of a carbohydrate-based hydrophilic and uncharged layer around the iron oxide

particles which imparts greater inertness. In addition, dextran coated MPs become more stable. The iron oxide particles coated with the higher amount of dextran (MP-DX-2) were selected to further proceed with ligand attachment.

Three different approaches were investigated for the attachment of boronic acid at the surface of the MP-DX-2 particles. The different magnetic supports produced were tested for binding to hIgG and BSA, resulting that APBA attachment through the GLYMO spacer yielded the best results (Table 2.1 and Figure 2.2A), as the affinity and specificity towards IgG was higher. Therefore, MP-BA3 particles were selected for further studies and characterization.

When comparing MP-BA3 particles with other MPs modified with synthetic ligands for antibody purification, these present competitive potential. The experimental adsorption values of ligand 22/8 modified MP having dextran and gum arabic coating was found to be 130 and 110 mg hIgG adsorbed/g MP respectively [16]. The commercially available APBA modified MP shows experimental adsorption of 109 mg hIgG adsorbed/g MP (Borlido et al. 2011), whereas MP-BA3 particles adsorb 173 mg hIgG/g MP.

The main differences between the commercial particles SiMAG-Boronic acid and the particles described in this work are: (1) commercial particles have an hydrodynamic diameter of 1 µm and ours possess particles with heterogeneous sizes between 200 nm to 2700 nm (Figure-2.4 A); (2) The core of the commercial particles is maghemite, whereas ours are magnetite; (3) the primary coating of our particles is porous silicate with dextran which differs from the commercial ones - SiMAG beads have an inert and stable non porous solid-phase silica matrix; (4) the method of APBA attachment onto commercial particles is not known and therefore it is not possible to compare with our data. As shown in Figure-2.1 (A) the different type of coating and functionalization methods and particles produced from them like MP-BA-1, MP-BA-2 and MP-BA-3 have wide variations in hIgG and BSA binding. Therefore, it is likely that the differences between the particles produced in this work and the commercial beads fully justify the differences observed. Dextran reduces non-specific binding and makes the magnetic support inert, as shown in this work and previously in [16], so that the actual binding was mainly through APBA coating on the surface.

The first characterization performed concerned the investigation of the correct structural displaying of boronic acid at the surface of MPs. Alizarin red (AR) is known to be a reporter to study carbohydrate-boronic acid interactions due to its change in fluorescence intensity upon binding to a boronic acid [20]. MP-BA3 particles were tested for binding and eluting alizarin red using two types of buffer conditions, previously shown to adsorb and elute IgG from commercial boronic acid particles [17]. From the results shown in Figure 2.2 (B), it is observed that for the binding with phosphate buffer pH 7.4 and elution with glycine buffer pH 11, MP-BA3 particles adsorbed ~40% AR and eluted ~30%. When binding and elution occurs at pH 8.5 with HEPES/Tris-HCl buffers, MP-BA3 particles adsorbed ~55% AR and eluted ~50%. For both buffer conditions tested, the control particles (MP-DX-2) bound a negligible amount of AR, indicating a correct display of the boronic acid moiety at the surface of the particles.

The selected MP-BA3 particles were further used to study the binding kinetics and the re-use potential. As shown in Figure 2D, the minimum time required for the maximum adsorption of IgG was estimated at 12 minutes, which was in accordance with the previous results obtained with commercial boronic acid particles [17], corroborating the fast kinetics of the interaction between APBA particles and the glycosylated IgG. Regarding the studies to ascertain the regeneration and re-use capability of the MP-BA3 particles, it was observed that until the fifth stage of recycle, particles retained about 75-80 % of protein binding capacity (when considering initial protein binding capacity as 100 %) (Figure 2.2 C).

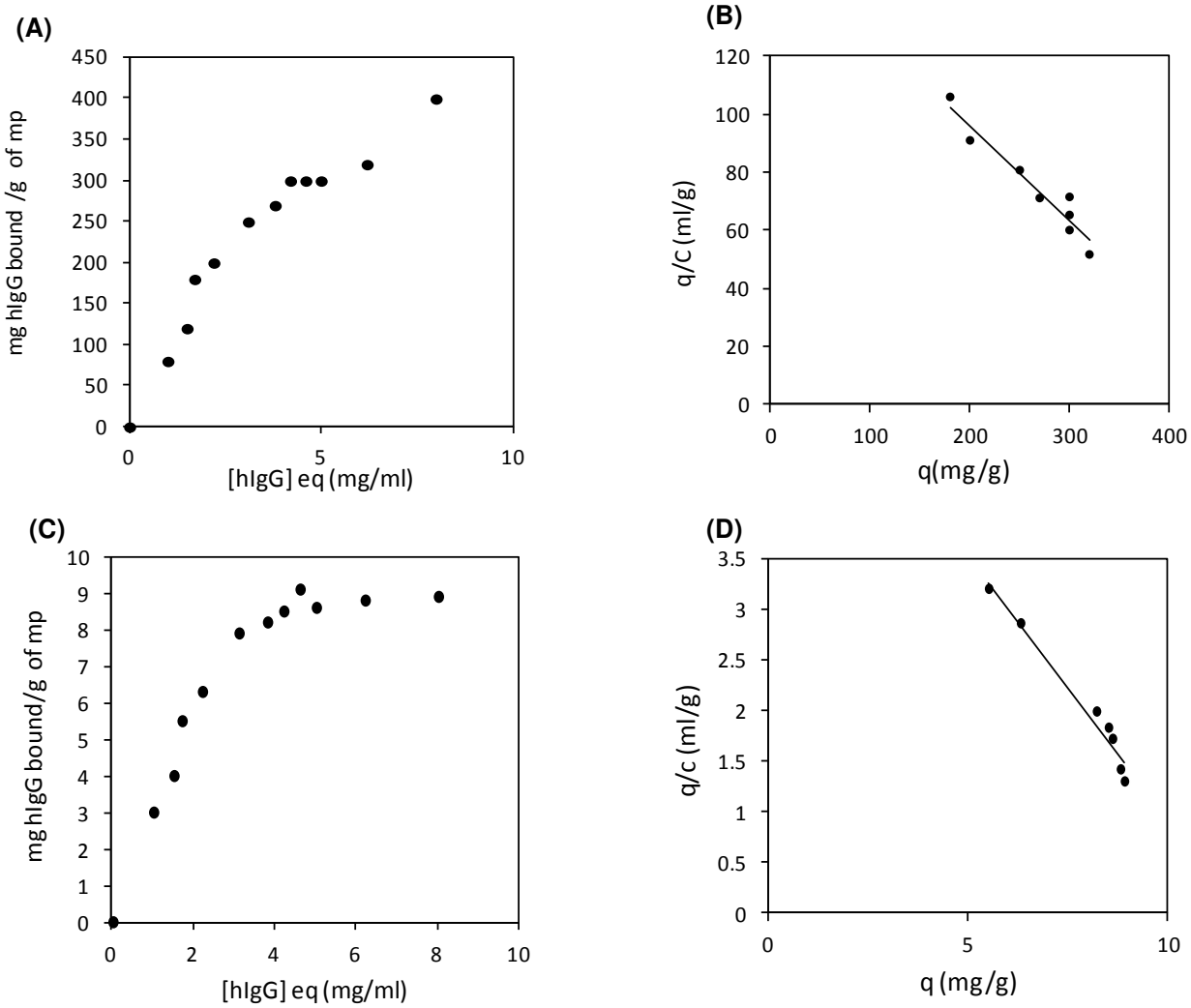


Figure: 2.3 Binding of human IgG at the surface of MP-Dex modified with APBA by GLYMO method MP-BA-3 (A; B) and MP-DX-2 Control (C; D). Representation of q (the amount of bound hIgG in equilibrium per mass of solid support) as function of C_{eq} (the concentration of hIgG in equilibrium). Experimental data was fitted with the expression q = (Qmax × Ceq) / (Kd + Ceq) using Scatchard plot,

where Q_{max} corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent), and K_d is the dissociation constant.

Finally, the static binding capacity of MP-BA3 particles was estimated by partition equilibrium experiments with pure solutions of hIgG. The adsorption isotherm of hIgG onto MP-BA3 could be fitted to a Langmuir profile yielding an affinity constant of $4.9\times10^4\ M^{-1}$ (K_a) and a theoretical maximum capacity of 492 mg hIgG adsorbed/g MP (Q_{max}) with a correlation factor of 0.93. For the control (MP-DX-2), which binds a negligible amount of hIgG, the determined theoretical maximum capacity was 3.1 mg hIgG adsorbed/g MP (Q_{max}). The values of K_a determined for MP-BA3 were lower than those estimated for the commercial boronic acid particles under the same experimental conditions, where the adsorption constant K was estimated as $1.5\times10^5\ M^{-1}$ when fitting a Freundlich isotherm to the experimental data set.

In terms of Q_{max} value (Table 2.2), the MP_Ga and MP_Dx modified with ligand 22/8 has a theoretical maximum capacity of 344 mg and 568 mg hIgG adsorbed/g MP [16], for ligand 22/8 on cellulose membrane the values were in the range of 630 mg hIgG adsorbed/g MP, the commercially available APBA MPs shows adsorption values 378 of mg hIgG adsorbed/g MP where as MPs as per this study shows Q_{max} 492 mg hIgG adsorbed/g MP.

2.5.2. Characterization of the magnetic materials for antibody adsorption

The selected magnetic support for IgG purification (MP-BA3) and the control unmodified particles (MP-Bare, MP-DX-2) were characterized by DLS to infer on the hydrodynamic diameter.

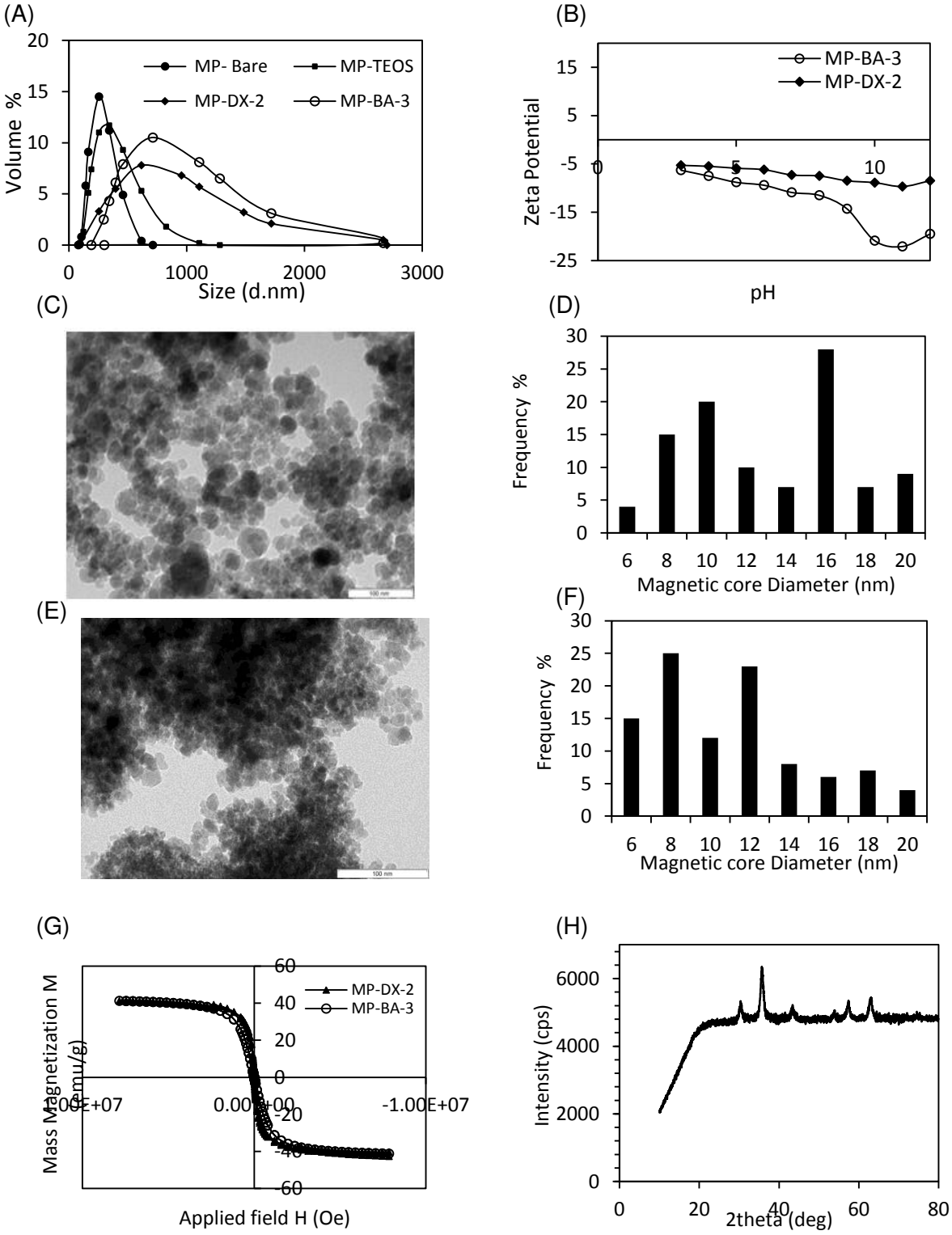


Figure. 2.4) Magnetic particles characterization by DLS for Particle size distribution of MP-Bare, MP-TEOS, Dextran(MP-DX-2) and APBA coated MP(MP-BA-3) (A); Zeta potential change of Dextran and APBA coated mp (MP-BA-3) with change of pH (B); TEM image of MP-BA-3 magnetic particles (C); grain size distribution from TEM for MP-BA-3 magnetic particles (D);TEM image of MP-DX-2 particles (E); grain size distribution from TEM for MP-DX-2 (F); VSM curves for MP-DX-2 and MP-BA-3 coated mp (G); and XRD spectra for MP-BA-3 (H).

As shown in Figure 2.4A, the average hydrodynamic diameter increased from 255-270 nm to 700-720 nm after dextran and APBA coating. In the later, the MP population is more heterogeneous in composition. The increase of MPs size upon biopolymeric coating has already been observed in other systems where gum arabic and dextran were employed [16]. In order to better control particle aggregation, synthesis and modification procedures could have been carried out with high speed agitation and ultrasound treatment. The separation of particles by size would be possible by application of magnets with increasing magnetic strengths or by HGMS. The particles used for bioseparation processes must balance the hydrodynamic diameter, related with the surface area, and the ease of separation. Very small magnetic nanoparticles (hydrodynamic diameters less than 200nm) are very difficult to separate with commonly available permanent magnets and also take a long time to separate (which can be deleterious to the protein particularly during elution steps); if using an electromagnet on high-gradient-magnetic-separation, small particles require high magnetic fields for separation (which contributes on its own for particle aggregation) and require a very tight control on the fluxes applied.

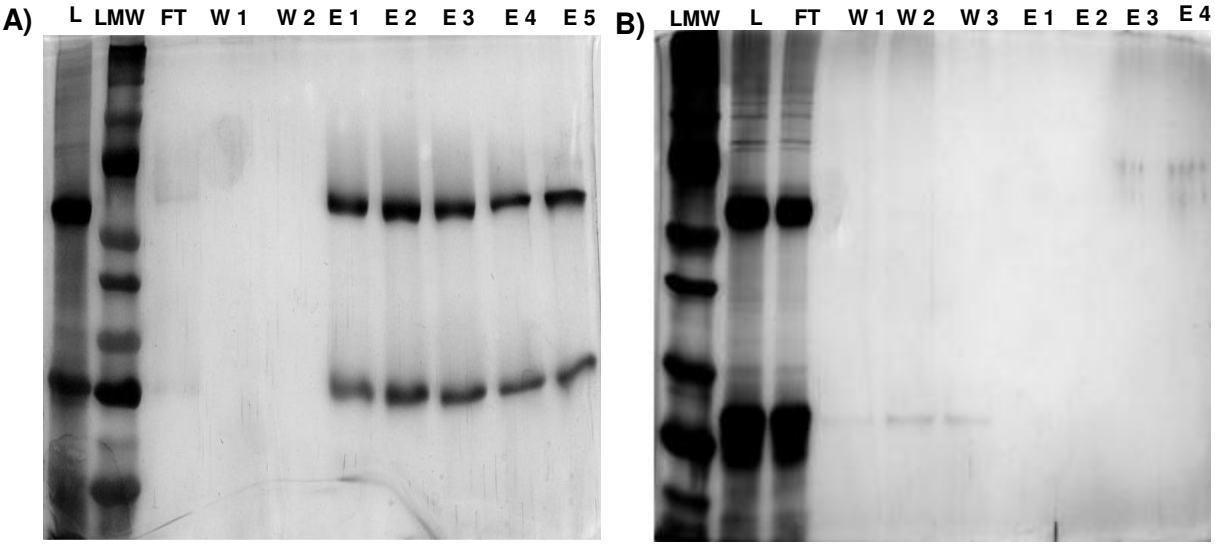
Regarding the variation of zeta potential with different pH values, results in Figure 4B indicate that control particles (MP-DX-2) have a zeta potential in the range of -5 mV at pH 3 varying gradually with the pH until reaching -9 mV at pH 11. In the case of MP-BA3, the zeta potential variation with pH is very similar to the behaviour observed for the control particles between pH 3 and 8 (Figure 2.4B), being -6.3 mV at pH3. However, from pH 8 onwards, and particularly when shifting from pH 9 to 10, there is considerable drop in the zeta potential values (from -14mV to -23mV). These results are associated with the transition from trigonal to tetrahedral structure at the pKa of boronic acids, estimated as 8.4 and 9.2 for free and immobilized phenyl boronic acids, respectively.

The characterization of particles by TEM confirmed the existence of spherical magnetic cores with an average diameter of 14 nm. Some heterogeneous population is also observed since the size distribution is between 6 – 20 nm (Figure 2.4C to F). With the VSM analysis it was possible to investigate the magnetic properties of the supports prepared. The curves shown in Figure 2.4G represent a typical no hysteresis curve with reversibility and symmetry, characteristic of the superparamagnetic behavior of the particles synthesized. In terms of saturation magnetization, the values obtained were 37 emu/g for MP-DX-2, and 34 emu/g for MP-BA2. The saturation magnetization value obtained for the MPs are consistent with the values referenced in [17] for commercial aminophenol boronic acid coated MPs having the range of 35 emu/g. The XRD spectra represented in Figure 4H shows, five characteristic peaks for Fe3O4,

marked by their indices ((2 2 0), (3 1 1), (4 0 0), (5 1 1), (4 4 0)). These peaks are consistent withdatabase ICDD 2007 (International Centre for Diffraction Data) and revealed that the resultants MPs were Fe3O4.

2.5.3. Tests with crude samples

After preliminary studies with pure solutions of hIgG and BSA, the MP-BA3 supports were tested with a CHO cell culture supernatant in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures.



C)

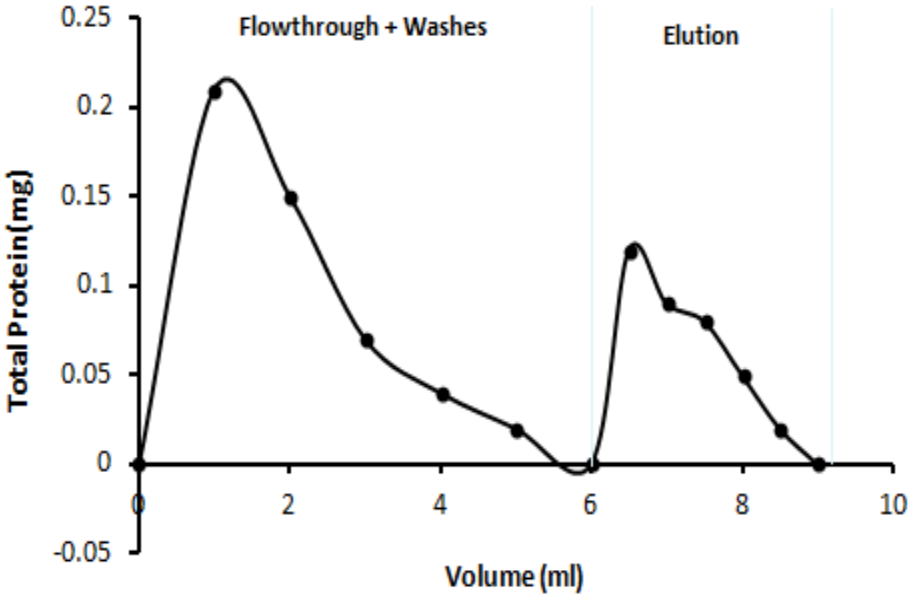


Figure: 2.5) Electrophoreses gel in denaturation conditions to verify the binding capacity as well as the best elution conditions for IgG from the GLYMO modified APBA coated mp (MP-BA-3) (A). LMW (low molecular weight); L (loading sample of the crude extract incubated with the adsorbent); FT (flowthrough); W1 (first wash with binding buffer-20mM HEPES pH 8.5); W2 (second wash with binding buffer); W3 (third wash with binding buffer); E1 (first elution-1M Tris-HCl, pH 8.5); E2 (second elution); E3 (third elution); E4 (fourth elution); E5 (fifth elution). (C) washes and elution profiles for total protein onto MP-BA-3.

Under reducing conditions, antibodies dissociate into several molecular weight structures of 100 kDa; 50 kDa and 25 kDa (Horák 2007). After confirmation by SDS-PAGE electrophoresis of the binding and elution capacity of the support on the crude extract used, the samples collected were quantified by the BCA method in order to determine the amount of protein bound to and eluted from the support and hence the purity of this recovery. From the BCA results it was possible to predict that approx. 91 % of total protein bound to the support. Using HPLC for quantification of IgG fractions it was observed that about 98 % IgG from crude sample bound to the magnetic particles and it is possible to elute 95 % of pure IgG at pH 8.5. The quantity of IgG eluted in five fractions was 21 %, 19 %, 20 %, 17 % and 18 % respectively.

2.6 Conclusions

Synthesis and study of MPs coated with APBA and modified using GLYMO led particles exhibiting promising characteristics for application in bioseparation processes. Human IgG purification will be most suiTable desirable option from a separation point of view. Synthesis and modification of magnetic support using the methods described in this study showed encouraging results in terms of production and utilization efficiency. The support showed superior performance under elution conditions despite the absorption of some (albeit a relatively small) quantity of undesired proteins. Nevertheless, the overall performance for human IgG separation was better than expected. One more advantage of this support is that, it showed low nonspecific adsorption in the presence of BSA and no major loss of the capacity of the support when reused up to five times. From these studies, it is possible to conclude that approx 98 % of the desired protein from crude extract was bound to the support, and at pH 8.5 using Tris-HCl elution conditions, it was possible to elute approx. 95 % desired protein based on adsorption.

References

1. Dias, A.M.G.C., Hussain, A., Marcos, A.S., Roque, A.C.A., 2011 A biotechnological perspective on the application of iron oxide magnetic colloids modified with polysaccharides. Biotechol. Adv. 29,142-155. (10.1016/j.bitechadv.2010.10.003)

2. Azevedo, A.M., Rosa, P.A.J., Ferreira, I.F., Pisco, A.M.M.O., Vries de J., Korporaal R.,Visser, T.J., Aires-Barros, M.R. 2009 Affinity-enhanced purificatin of human antibodies by aqueous two-phase extraction. Sep.Purif.Technol. 65,31-39. (doi:10.1016/j.seppur.2008.03.006)

3. Kuczewski, M.., Schirmer, E., Lain B., Gregory, Z. 2011 A single-use purification process for the production of a monoclonal antibody produced in a PER.C6 human cell line. J. Biotech. 6,56-65. (doi: 10.1002/biot.201000292)

4. Lattuada, Marco., Alan Hatton, T. 2007 Functionalization of Monodisperse Magnetic Nanoparticles. Langmuir. 23,2158-2168. (doi: 10.1021/la062092x)

5. Motornov, M., Roiter, Y., Tokarev, I.., Minko, S. 2010 Stimuli-responsive nanoparticles, nanogels and capsules for integrated multifunctional intelligent systems. Prog. Poly. Sci. 35,174-211. (doi: 10.1016/j.progpolymsci.2009.10.004)

6. Hao, R., Xing, R., Xu, Z., Hou,Y., Gao,S., Sun,S. 2010 Synthesis, Functionalization, and Biomedical Applications of Multifunctional Magnetic Nanoparticles. Adv. Materials. 22, 2729-2742(doi:10.1002/adma.201000260)

7. Kim, J., Kim, H.S., Lee, N., Kim, T., Kim, H. 2008 Multifunctional Uniform Nanoparticles Composed of a Magnetite Nanocrystal Core and a Mesoporous Silica Shell for Magnetic Resonance and Fluorescence Imaging and for Drug Delivery. Angewandte Chemie International Edition. 47**,** 8438-8441. (doi : 10.1002/anie.200802469)

8. Liu, S., Chen, H., Lu, X., Deng, C., Zhang, X., 2010 Facile Synthesis of Copper(II)Immobilized on Magnetic Mesoporous Silica Microspheres for Selective Enrichment of Peptides for Mass Spectrometry Analysis. Angewandte Chemie International Edition.49,7557-7561. (doi: 10.1002/ange.201003602)

9. Kelley, B. 2009 Industrialization of mAb production technology: The bioprocessing industry at a crossroads. MAbs.1, 443-452

10. Hao, R., Yu, J., Hou, Y., Sun, S., 2011 One-pot synthesis of hollow/porous Mn-based nanoparticles via a controlled ion transfer process. Chem. Commun. 47, 9095-9097. (doi: 10.1039/c1cc12759d)

11. Yang, C., Wub, J., Hou, Y., 2011 Fe3O4 nanostructures- synthesis, growth mechanism, properties and applications. 47, 5130-5141. (doi: 10.1039/c0cc05862a)

12. Chen, C.T., Chen,Y.C. 2010 Functional magnetic nanoparticle-based label free fluorescence detection of phosphorylated species. Chemical Comm.46,5674-5676. (doi:10.1039/C0CC00637H)

13. Xu, Z., Shen, C., Hou, Y., Gao, H., Sun, S., 2009 Oleylamine as Both Reducing Agent and Stabilizer in a Facile Synthesis of Magnetite Nanoparticles. Chemistry of Materials. 21, 1778–1780.(doi: 10.1021/cm802978z)

14. Gomes, A.G., Azevedo, A.M., Aires-Barros,M.R., Prazeres, D.M. 2011 Studies on the adsorption phenyl boronic acid. J.Chromatogr A, 1218, 8629-8637. (doi: 10.1016/j.chroma.2011.10.004)

15. Ralla, K., Anton, F., Scheper, T., Kasper, C., 2009 Application of conjoint liquid chromatography with monolithic disks for the simultaneous determination of immunoglobulin G and other proteins present in a cell culture medium. J.Chromatogr A, 1216, 2671-2675. (doi: 10.1016/j.chroma.2008.09.102)

16. Sara Santana, D. F., Vijaykumar, L.D., Roque, Ana C.A. 2012 Dextran coated magnetic supports for antibody purification. Appl. Mater. Interfaces,. 4,5907-5914.(doi: 10.1021/am301551)

17. Borlido, L., Roque, Ana C.A., Aires-Barros M.R. 2011 Potential of boronic acid functionalized magnetic particles in the adsorption of human antibodies under mammalian cell culture conditions. J.Chromatogr A,. 1218,7821-7827. (doi: 10.1016/j.chroma.2011.08.084)

18. Lin,Z.A., Zheng, J.N., Lin, F., Zhang, L., 2010 Synthesis of magnetic nanoparticles with immobilized aminophenylboronic acid for selective capture of glycoproteins. J. Material Chemistry,. 21, 518-524. (doi: 10.1039/c0jm02300k)

19. Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Vander, E.L., Muller, R.N., 2008 Magnetic Iron Oxide Nanoparticles: Synthesis, Stabilization, Vectorization and Biological Applications. Chemical Reviews, 108, 2064-2110. (doi: 10.1021/cr068445e)

20. Springsteen, G., Gregory, W.B., 2001 Alizarin Red S. as a general optical reporter for studying the binding of boronic acids with carbohydrates. Chem Commun (Camb), 17, 1608-1615. (doi: 10.1039/b104895n)

CHAPTER 3

PROCESS INTEGRATION OF AQUEOUS TWO PHASE EXTRACTION WITH MAGNETIC SEPARATION

SUMMARY

The objective of this work is to assess the capability of process integration for enhanced and effective antibody purification. It also evaluate the use of different ATPSs based on PEG/Dextran and supplemented with magnetic particles modified with amino phenyl boronic acid. The basic core of magnetic particle was first coated with different biological and organic polymers and then used for modification. In order to compare the integrated magnetic separation-aqueous two phase extraction to the ATPE alone, the first studies were performed using basic core coated with biopolymer. The effect of ionic strength, pH, polymer molecular weight and polymer weight percentage on pure IgG partition was evaluated.

This promising technology in combination with magnetic particles can overcome some of the technical drawbacks currently encountered using the established purification platform, such as cost, productivity and diffusional limitations.ATPSs are formed by joining two incompatible polymers, or one polymer and a salt, above certain concentrations. The partition of substances between two phase aqueous systems is determined by complex phenomenon such as van der waals forces, hydrogen bonds, charge interactions, hydrophobic interactions and steric effects.

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Application Number: 107379 J

3.1. Introduction

The separation and purification of biopharmaceutical products are important factors from commercialization point of view based on customer demand and number of applications for treatment of diseases. Existing research work and methodologies represents that separation methods costs for 75 % [1] of the total production cost especially for products like proteins [2], antibodies [3] and enzymes [4].

The important reason behind higher cost was associated with technologies that were used for processing of these products [5]. Sensitivity of biomolecule plays important role from separation point of view [6] which will affect usually antibodies, proteins and enzymes which are sensitive molecules for temperature as well as processing conditions [7] responsible for making processing more difficult [8]. These molecules and its three dimensional structure get easily denatured [9] and loose its activity as well as applicability [10] by extreme pH [11], temperature [12], pressure [13] , solvents [14] as well as exposure to air [15].Those molecules that do not get damaged by above mentioned extreme conditions were easier to process and processing cost easily gets reduced [16]. Also from application point of view for clinical use these products needs to be in ultrapure form [17] . During synthesis of these compounds by fermentation process they were produced in very less concentrations [18] which contain highly mix composition of biomolecules [19], impurities and undesired components [20] along with cells which needs to be removed [21]. These impurities removal [22], suspended solids separation [23], product concentration followed by purification [24] is responsible for several bioprocessing steps which makes process more complex as well as costly [25]. Important components of bioseparation processes includes removal of cell components and fragments [26], product separation [27] and concentration, product purification to obtain higher purity product and polishing [28] which makes product acceptable for clinical and therapeutic applications [29].

Improved development strategies were mainly associated with reduction of product residence time [30] as well as downstream processing steps to enhance product yield and product quality[31]. With these strategies new trend come into existence which involves combination of multiple techniques to create hybrid technologies [32].Based on production and processing capacities bioprocesses were categorised in three main sections [33]. Processes whose process volume exceeds 100,000 L per batch and have superior separation strategies considered as 1arge-scale processes [34]. Products such as ethanol, polysaccharides, acetic acid and acetone are categorized in this type [35]. Medium scale processes involve antibiotics production [36] whose processing is quite robust and from cost point of view final cost depends on the initial volume reduction steps [37]. Small scale processes includes production of antibodies and enzymes [38] for therapeutic applications, usually fragile and delicate molecules are responsible for increase in separation and purification cost [39]. New type of hybrid separation and purification technologies plays important role in isolation of these types of biomolecules [40] in higher purity and major research attention is provided by research community to develop much better and efficient techniques. These small scale bioseparations are the most difficult and costly which is still biggest challenge for making these products cheaper and beneficial from customer point of view [41].

3.2. Experimental

3.2.1. Materials

Sodium silicate ($Na_2SiO_3.5H_2O$), Amino phenyl boronic acid (APBA), Tris(Hydroxymethyl)amino methane, Dextran from *Leuconostoc mesenteroides*, HEPES, Glutaraldehyde, Tetraethoxy Silane (TEOS), Gum arabic, Anthrone, Sodium Hydroxide, 3-Glycidyloxypropyl trimethoxysilane (GLYMO), Ferric sulfatehydrate $[Fe_2(SO_4)_3].H_2O$, Ferrous sulphate heptahydrate ($FeSO_4.7H_2O$), (3-Aminopropyl) triethoxysilane (APTES), and Alizarin red were purchased from Sigma – Aldrich.

Poly (ethylene glycol) with molecular weight 3,350 and 8,000 was purchased from Sigma (St. Louis, MO, USA). Poly (ethylene glycol) with molecular weight 1,000, 3,500, 6,000 and 10,000 was purchased from Fluka (Buchs, Switzerland). Dextran with an average molecular weight of 500,000 was purchased from Fluka (Buchs, Switzerland). The reagents used for SDS-PAGE Gels were, Ammonium Persulphate (APS), N,N,N,N–Tetramethylethylenediamine (TEMED), 30% Acrylamide/Bisacrylamide-solution, SDS micropellets (Sodium dodecyl sulphate), Silver Stain Plus Kit purchased from BIO-RAD.

Polyclonal human immunoglobulin G (IgG) for therapeutic administration (Gammanorm) was purchased from Octapharma, as a 165 g/L solution containing 95% of IgG. A Chinese Hamster Ovary (CHO) cell supernatant containing monoclonal human IgG directed against interleukin-8 were developed by BERG group at tagus park research facility.

3.2.2. Magnetic particles Synthesis

The process of synthesis of nanoparticle with polymer coating consists of dissolution of 2.36 g FeSO4.7H2O and 6.79 g Fe2(SO4)3.5H2O in 150 ml deionized water, which was preheated to 80^0C before the coprecipitation reaction. When the temperature reaches to 80 degree centigrade then 10 ml ammonium hydroxide was added and vigorously stirred for 30 minutes at 1200 rpm. After 30 minutes the resulting nanoparticles are collected using magnet and thoroughly wash d several times with deionized water.

3.2.3. Silica modification of magnetic microspheres

In the next step of silica coating 120 ml aq. Solution containing 3.4 g Na2SiO3.5H2O and 0.4 g NaOH was introduced into the resultant particles. Then the particles along with solution are ultrasonicated for 5 min. After that 2M HCl was added dropwise into the dispersion to adjust the pH to 6. Then, the particles coated with a thin SiO2 layer were washed with deionized water and collected by applying an external magnetic field.In the further step to obtain porous silica coating on the surface of magnetic nanoparticles a sol-gel process is used. In this process step above prepared particles were dispersed in a mixture of 50 mL ethanol, 50 mL deionized water and 1mL concentrated ammonia solution with the help of ultrasonication, and a stable dispersion was obtained. Subsequently, 200 mL TEOS dissolved in 10 mL ethanol was added to the above dispersion at a rate of 1 mL per min under mechanical stirring and the reaction was allowed to proceed at 850C for 0.5 h. In this way TEOS coated nanoparticles were obtained.

3.2.4. Polymer coating of magnetic nanoparticles

Magnetic nanoparticles coated with silica are highly prone to non-specific adsorption and hence there is requirement to provide such a coating of polymers which will avoid this problem of undesirable protein adsorption with these requirement magnetic nanoparticles are coated with polymers like Dextran-500000,Gum Arabic,CM-Dextran,PEG-3500, EOPO, Jeffamine-M-2070, Polyacrylic acid sodium salt-15000, UCON-2000, UCON-3520 respectively. In this polymer coating process TEOS coated mnp are stirred mechanically with solution of biopolymer to obtain desired biopolymer coated mnp.

3.2.5 Aqueous Two-Phase Extraction Studies with MPs

Aqueous two-phase systems were prepared by weighting the corresponding stock solutions of PEG and Dextran polymers with salt in order to achieve the desired final composition of each system. Pure IgG extraction studies were performed by adding 1 mL of 1 g/L IgG stock solution. In IgG extraction studies from CHO cell culture (1.16 g/L IgG) the supernant loading ranged from 1 to 1.5 ml. All systems were prepared in 15 mL graded test tubes to a total final weight of 5 g by adding water (Milli-Q), PEG 3350 Da stock solution (40 %), dextran 500,000 Da stock solution (20 %),MP at a final concentration of 0.02% (w/w) and salt in the concentration range of 100-500 mM. IgG extraction studies were performed by thoroughly mixing each system components in a vortex shaker, after which phases were left to separate for 2 - 4h at room temperature (~25°C). After phase separation, the test tubes were positioned on a magnetic separator,samples of each of the phases were taken and the magnetic particles were collected. The recovered particles were washed five times, the first one with Mili-Q water and then four consecutive washes with 20 mM HEPES buffer at pH 8.5 (0.5 ml volume each wash). Elution of adsorbed IgG was then triggered using 1.5 M Tris-HCl buffer at pH 8.5, and MPs were washed with this buffer 5 times(0.5 ml volume of each washing). The quantity of IgG in both bottom and top phases as well as from MPs were analysed by HPLC, whereas total protein was assayed by the Bradford method and purity assessed by SDS polyacrylamide gel electrophoresis.

For the study of the best incubation period between GA-APBA-MPs and a pure human IgG solution, the same protocol has described above was followed with the exception that IgG extraction studies were carried out by thoroughly mixing each system components in a vortex shaker for 10, 15, 20, 25 or 30 minutes, followed by phase separation at room temperature. In order to assess the recycling properties of the GA-APBA-MPs, up to five cycles of magnetic ATPSs were carried out with the same particles by regenerating them after each round through a washing with regeneration buffer (0.1M NaOH, 30% (v/v) isopropanol).

3.3. Analytical Methods

3.3.1. Protein A chromatography

Äkta Purifier system from GE Healthcare (Uppsala, Sweden) was used for measurement of IgG concentration in the top and bottom phases to carry out analysis. Quantification was done by using porous protein A affinity column from Applied Biosystems (Foster City, CA, USA). IgG concentration was

determined from a calibration curve obtained using Gammanorm IgG as a standard.

3.3.2. Protein quantification

Bradford method was used to determine total protein concentration using a Coomassie reagent from Pierce (Rockford, IL, USA). The standard for protein calibration was made with bovine gamma globulin. Absorbance was measured at 595 nm in a Spectra Max 340PC microplate reader from Molecular Devices (Sunnyvale, CA, USA).

3.4. Characterization of Magnetic Particles

3.4.1. Zeta Potential

Zeta potential measurements were performed with a Zetasizer Nano ZS system from Malvern (Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with different pH solutions of 10 mM KNO3, ranging from pH 3 to 12. The zeta potential was calculated using the Smoluchowski equation.

3.4.2. Hydrodynamic Diameter

Hydrodynamic diameter measurements were performed with a Zetasizer Nano ZS system from Malvern (Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with milli-Q water and then used for measurement.

3.5. Results and discussion

3.5.1. Adsorption Isotherms of Polymer coating on magnetic particles

In order to study the quantity of biopolymer that we able to coat on the surface of magnetic nanoparticles adsorption isotherm study was carried out. The results of adsorption isotherm on Bare mnp were shown in Figure-1 and on TEOS mnp were shown Figure- 2. The maximum quantity of Dextran, Gum Arabic and CM-Dextran we able to coat on the surface of bare mnp were 1.4, 1.3 and 1.1 mg per gm of particles respectively. In case of TEOS coated particles there was gradual rise in quantity of biopolymer that was coated on the surface. It was observed 2, 1.8 and 1.1 mg per gm of particles coating for Dextran, Gum arabic and CM-Dextran respectively. In case of organic polymers it was difficult to perform adsorption isotherm study because of lack of suitable quantification technique for adsorption measurement. The morphology study using TEM, Charge on particles, Particle size and antibody adsorption experiments were used as major tools for analyzing organic polymers.

Usually basic magnetic core of MPs has inert surface and too less target oriented applicability. Electrostatic adsorption and repulsion plays important role in utilization of magnetic particles for specific separation. The major challenge of magnetic particles when we have to use them for selective adsorption and desorption point of view in IgG purification industry is non-specific binding. The crucial challenge of non-specific binding can be reduced to higher extent by coating of the iron oxide particles with polymers, because it reduces the nonspecific interactions when compared with bare MPs. The major reason behind reduction in nonspecific binding is the creation of a more hydrophilic layer around the iron oxide particles which is responsible to make the magnetic support more inert (Hermanson et al. 1992). As far as organic polymer coating is concerned magnetic particles coated with Polyacrylic acid sodium salt-15000 and

UCON-3520 shows effective inertness to non-specific binding in case of biopolymers coated MPs Dextran-500000 found to be inert. The major reason behind having superior inertness using Polyacrylic acid sodium salt-15000,UCON-3520 and Dextran was nature of polymer which makes particles surface less reactive. After successful coating of mp using polymers it is also important to keep in mind that actual coating on surface is not uniform and homogeneous which expose the reactive iron oxide particle for interactions with other particles while use and cause the nonspecific adsorption of support. The stability and inertness results of polymer coated MPs provide best opportunity to utilize these particles for aqueous two phase extraction process with further functionalization using artificial ligand or target specific organic compounds like boronic acid.

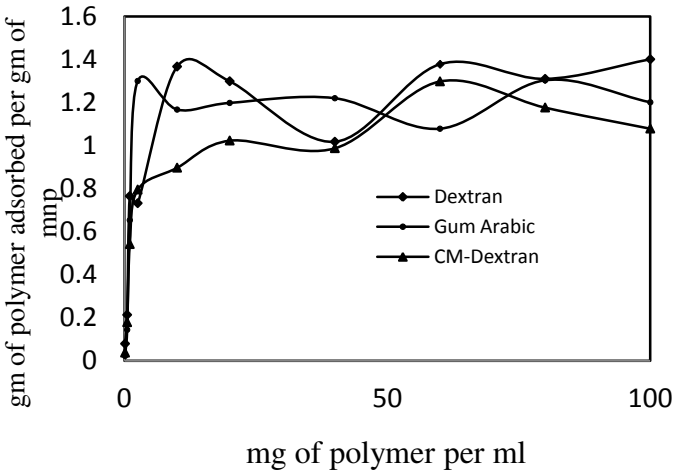

Figure.3.1. Adsorption isotherm of Dextran, Gum Arabic and CM-Dextran in gm of polymer adsorbed per gm of bare MNP

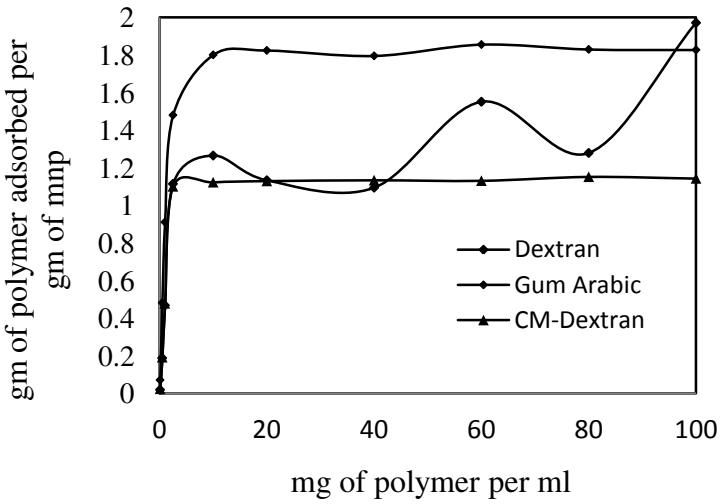

Figure.3.2. Adsorption isotherm of Dextran, Gum Arabic and CM-Dextran in gm of polymer adsorbed per gm of TEOS coated MNP

3.6 Aqueous Two Phase extraction process with bio and organic polymer coated MPs

The partition of pure IgG in PEG / Dextran ATPSs supplemented with different polymer coated MPs was evaluated. In order to increase IgG quantification by chromatography easier and more accurate, it was added with pure IgG (from Gammanorm) having concentration of 1 gm/L. As per the results shown in figure 3.3 A, 3.3 B and 3.3 C salt concentration plays important role in IgG partitioning along with magnetic particles. Higher yields of IgG in the upper phase are obtained using high concentrations of NaCl. The salt concentration has been studied for 100, 200,300,400 and 500 mM of NaCl. It was observed that by increasing salt concentration there is decrease in the concentration of IgG in the lower dextran phase and a simultaneous increase in the concentration of IgG in the upper PEG-rich phase.

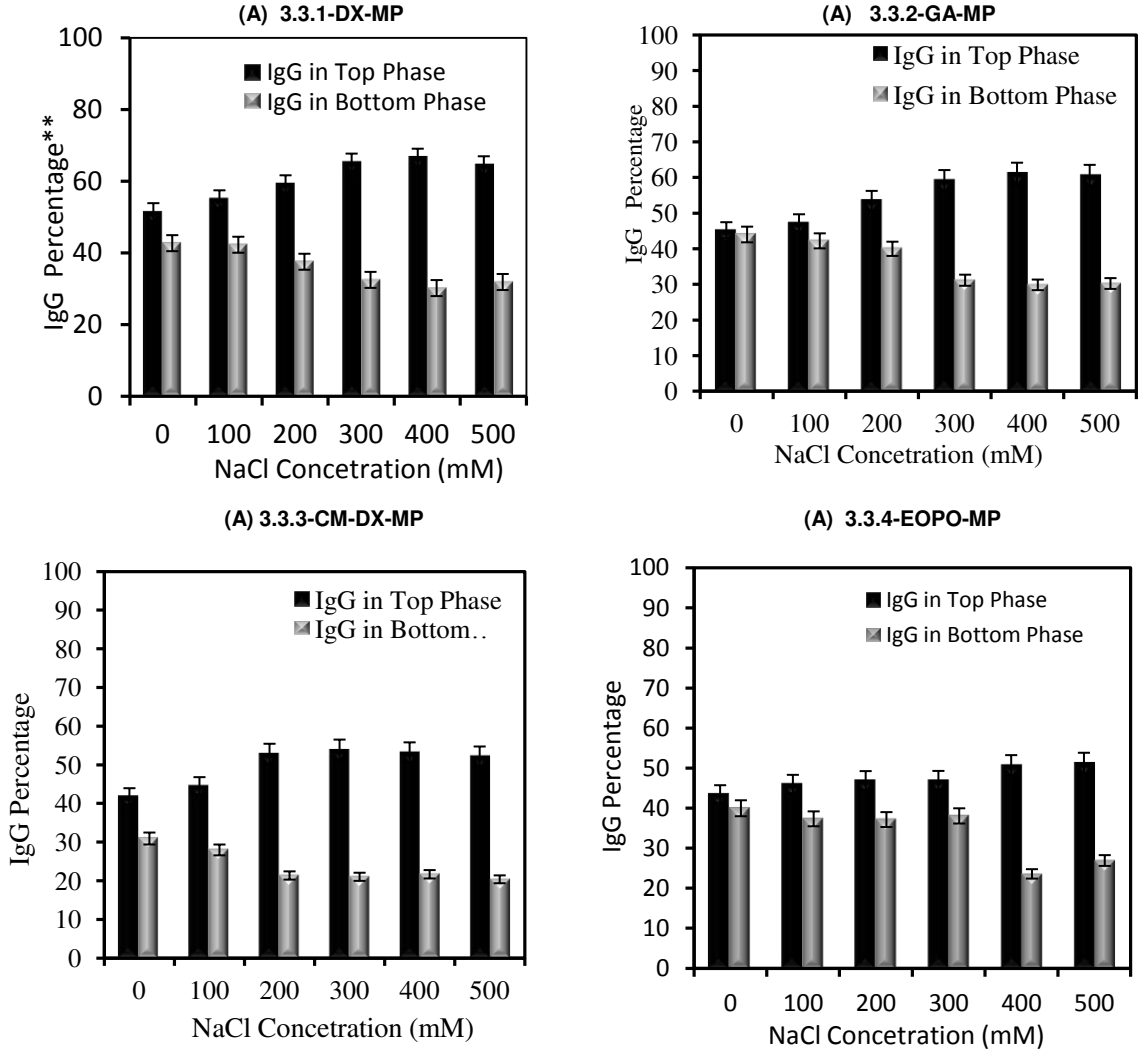

Figure.3.3 (A) Pure IgG extraction parameters in PEG/Dextran system with organic and biopolymer coated MPs for increasing NaCl Concentrations Dextran(3.3.1-DX-MP), GumArabic (3.3.2-GA-MP), CM-Dextran (3.3.3-CM-DX-MP) and EOPO-MP(3.3.4).

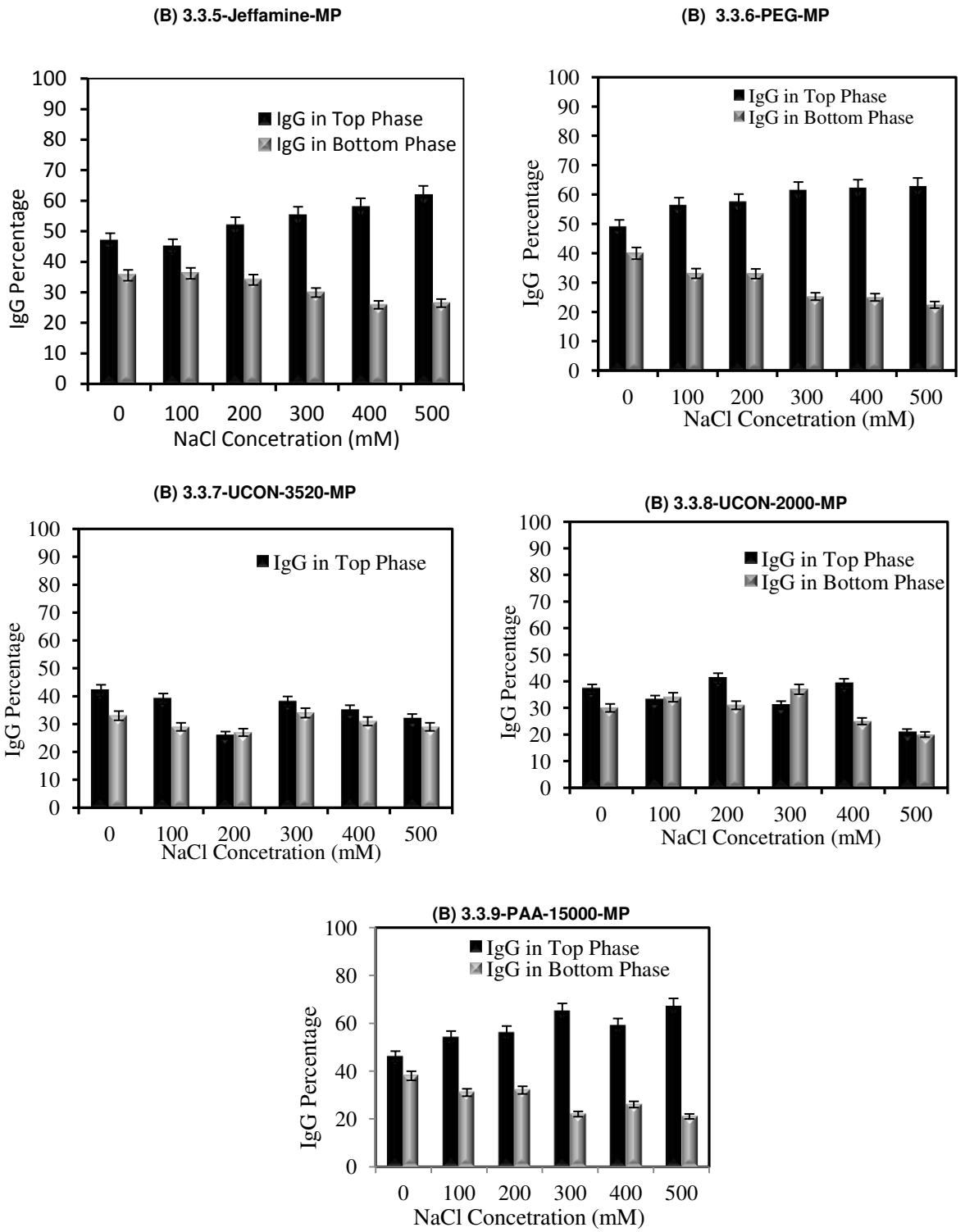

Figure.3.3 (B) Pure IgG extraction parameters in PEG/Dextran system with organic and biopolymer coated MPs for increasing NaCl Concentrations Jeffamine-MP(3.3.5), Polyethylene Glycol(3.3.6-PEG-MP), UCON-3520-MP(3.3.7), UCON-2000-MP(3.3.8), and Polyacrylamide sodium salt(3.3.9-PAA-15000-MP).

3.7 Antibody Partition coefficients of bio and organic polymer coated MPs

The IgG partition coefficients in ATPS systems show that antibody distribution to top phase increases when NaCl concentration is greater than 200 mM. The interactions between the antibody and the polymer coated particles are non-specific, hydrophobic and ionic. Addition of salt is responsible for stronger interactions between magnetic particles and IgG whereas an ionic interaction becomes strong. Rise in salt concentration is responsible for decrease in electrostatic potential difference between the upper and lower phase because of even distribution of chloride ions between both the phases. These favourable electrostatic interactions also present in upper phase between IgG and chloride ions.

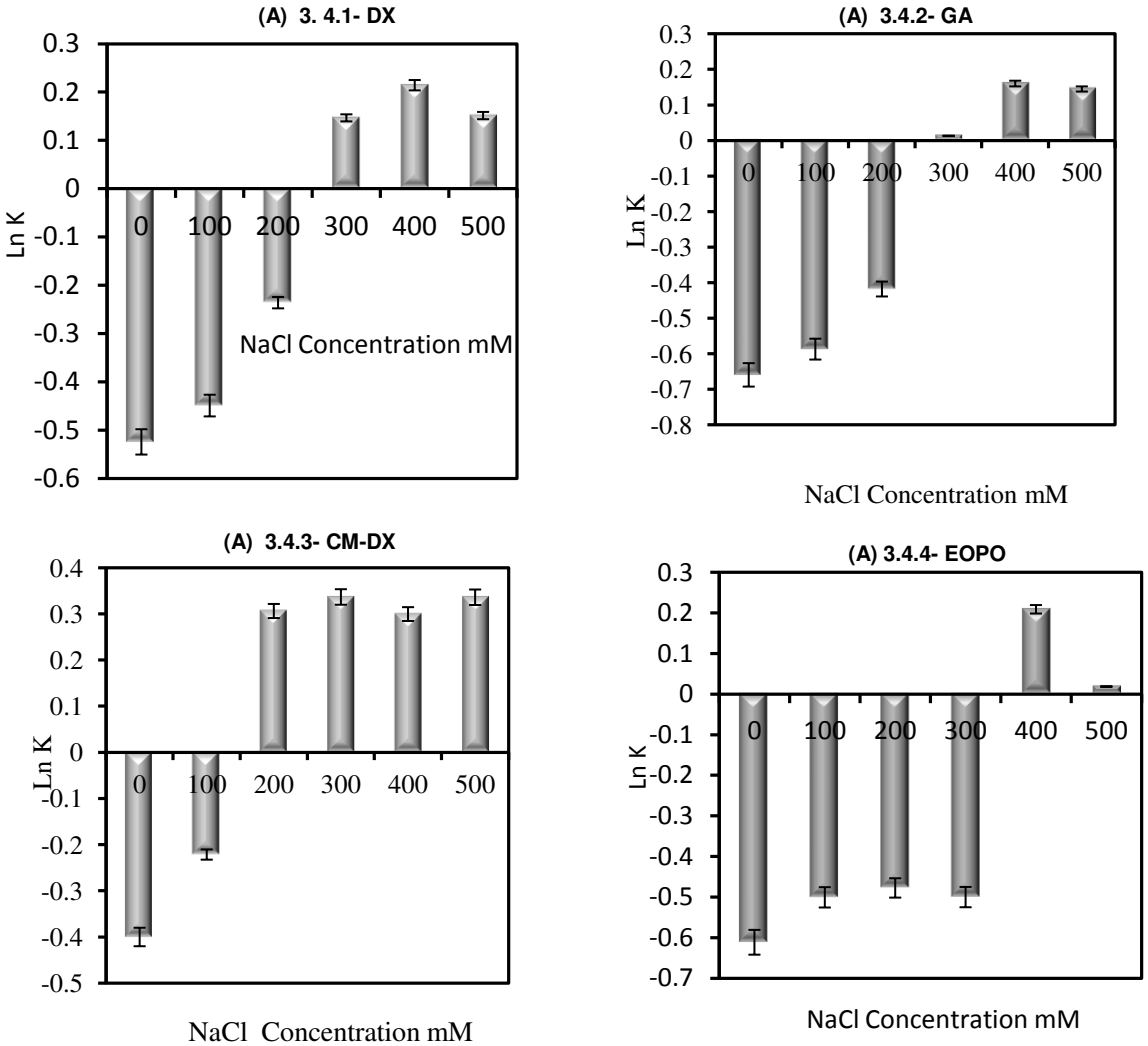

Figure: 3. 4 (A) Logarithum of the pure IgG partition coefficient in systems containing organic and biopolymer coated MPs and with increasing NaCl concentration. Dextran(3.4.1-DX-MP), GumArabic (3.4.2-GA-MP), CM-Dextran (3.4.3-CM-DX-MP), EOPO-MP(3.4.4).

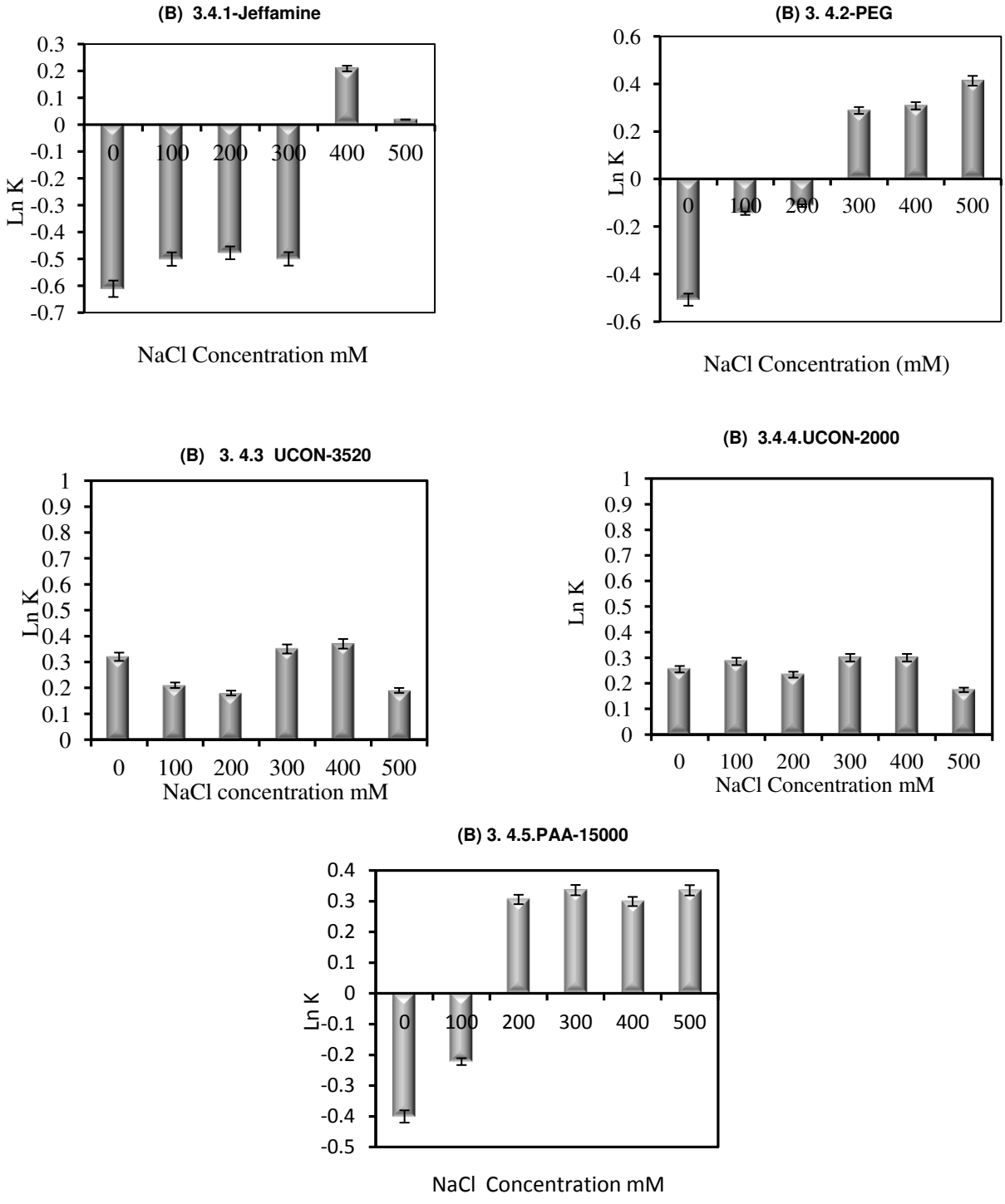

Figure: 3. 4 (B) Logarithum of the pure lgG partition coefficient in systems containing organic and biopolymer coated MPs and with increasing NaCl concentration. Jeffamine-MP(3.4.5), Polyethylene Glycol(3.4.6-PEG-MP), UCON-3520-MP(3.4.7), UCON-2000-MP(3.4.8),and Polyacrylamide sodium salt (3.4.9-PAA-15000-MP).

3.8 IgG binding and elution studies

The IgG binding studies results were shown in figure- 3.5. IgG elution studies from polymer coated particles were performed by testing different eluents in particles into which IgG was previously adsorbed. As the desired target MPs should have low IgG adsorption capacity, elution is important to know how much IgG particles adsorbed in adsorption step and at which proportion they elute.If basic core adsorb IgG after functionalization with ligand or desired chemical compound on the its surface it is extremely difficult to elute the IgG during the processing so it is highly essential to have adsorption inert core.

After binding and elution capacity study all the samples collected were quantified by Protein-A HPLC in order to determine the amount of protein bound to and eluted from the support. From the HPLC results (Figure-3.5) it was possible to predict that about Dextran,Gum Arabic,PEG and PAA-15000 were the best support as they bind less quantity of IgG.

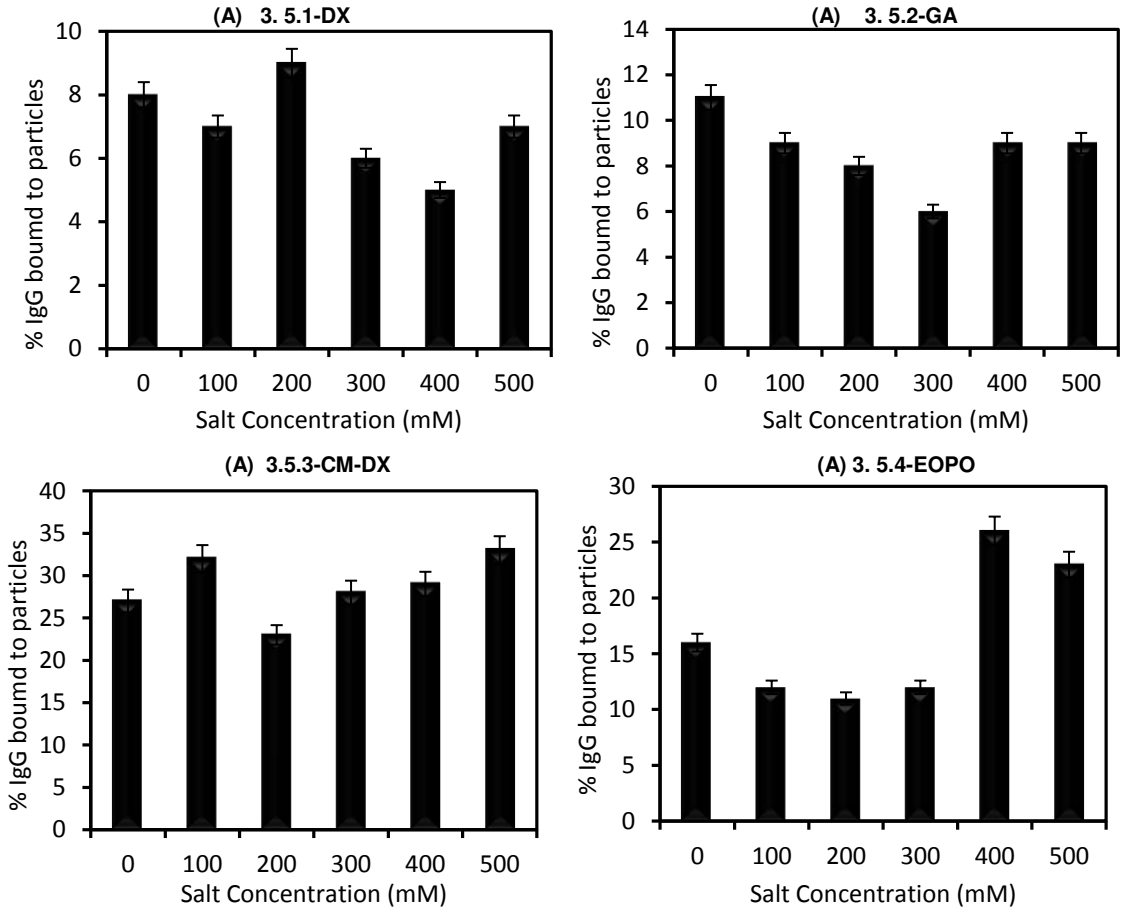

Figure 3.5 (A) Percentage of pure IgG bound to magnetic particles with increasing NaCl concentration as Dextran(3.5.1-DX-MP), GumArabic (3.5.2-GA-MP), CM-Dextran (3.5.3-CM-DX-MP), EOPO-MP(3.5.4).

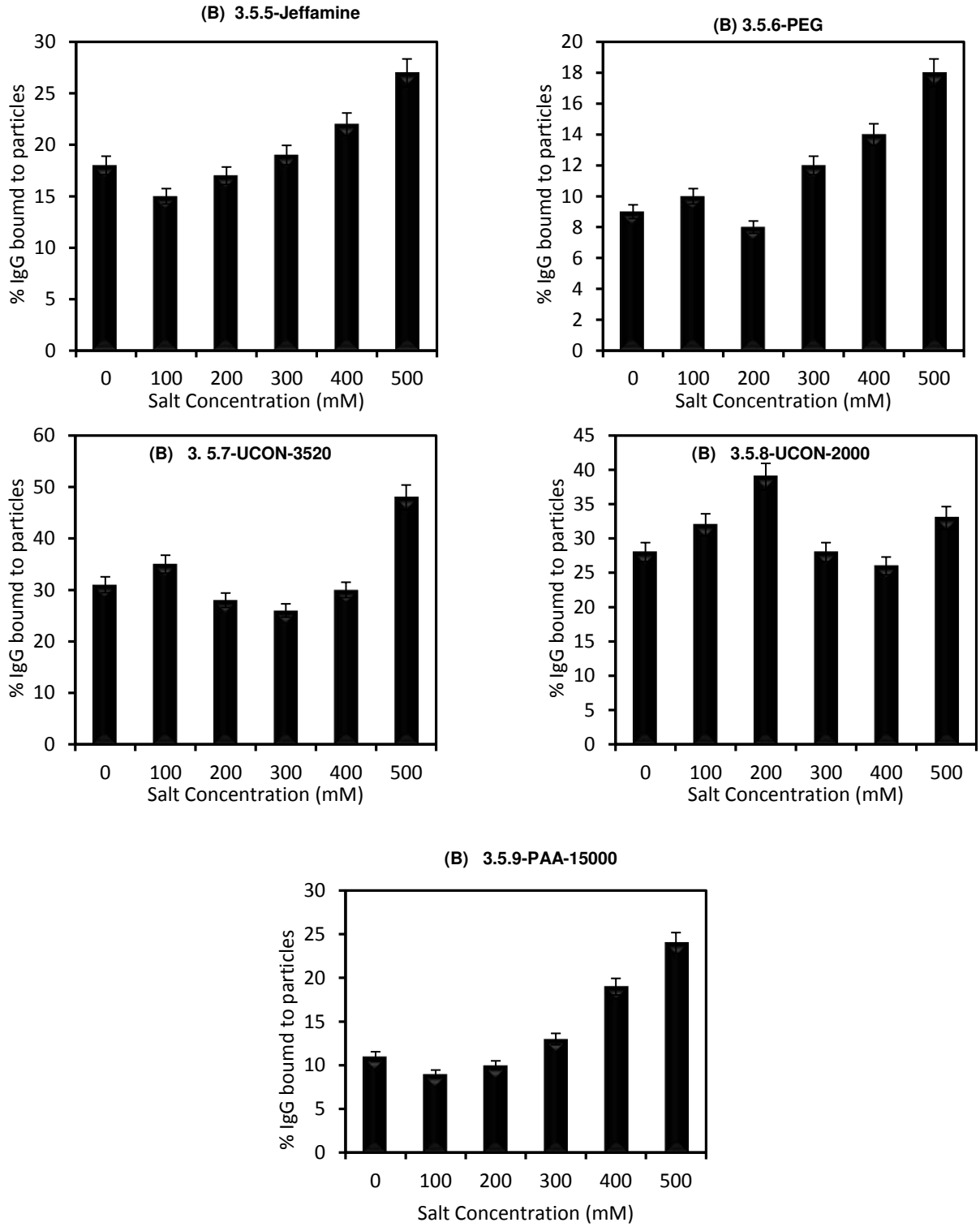

Figure 3.5 (B) Percentage of pure IgG bound to magnetic particles with increasing NaCl concentration as Jeffamine-MP(3.5.8), Polyethylene Glycol(3.5.6-PEG-MP), UCON-3250-MP(3.5.4), UCON-2000-MP(3.5.5), and Polyacrylamide sodium salt(3.5.9-PAA-15000-MP).

3.9. Particle size distribution of magnetic particles

Particle size plays important role in phase segregation as well as for good performance of magnetic particle in aqueous two phase system. The modifications using organic as well as biopolymers increases the hydrodynamic diameter of the particle. As shown in figure-3.6,the average hydrodynamic diameter for PEG Coated MNPs, is around 585 nm. The average hydrodynamic diameter for bare mnp varies in the range of 210-245 nm. In case of particles coated with triethoxy silane rise in average hydrodynamic diameter observed to 290-325 nm. The mp with dextran-500000 have average diameter in the range of 550-575 nm, eventhough some particles of size upto 3000 nm are also observed in the suspension.The particles coated with gumarabic also shows particle size in the range of 530-540 nm.UCON-3250 coated MP shows higher particle size in the range of 1650-1700 nm.Particles of CM-Dextran and UCON-2000 are of quite similar particle size range.EOPO coated magnetic particles also have higher range of particle size around 1000-1050 nm.

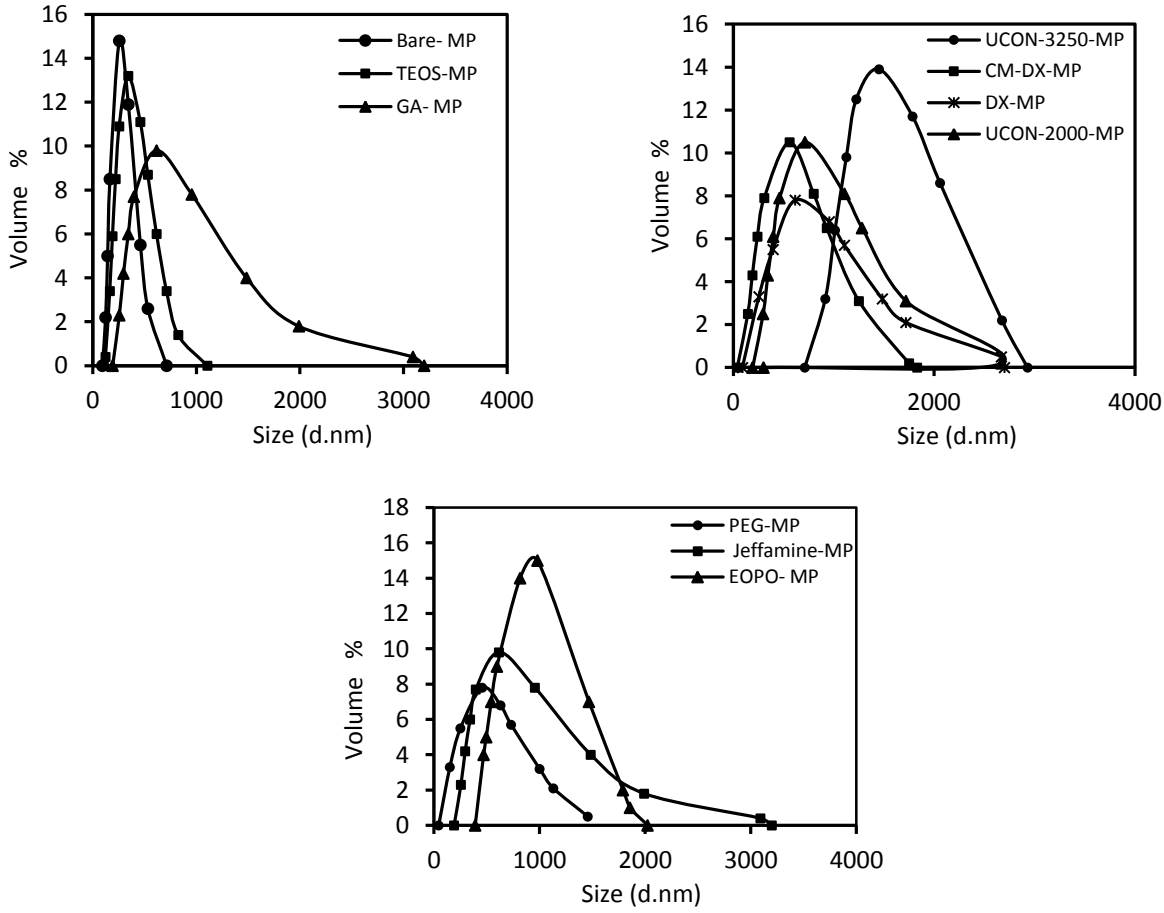

Figure.3.6. Magnetic particles Dynamic light scattering results for Particle size distribution of Bare-MP, TEOS-MP, Gum Arabic(GA-MP), Dextran(DX-MP), CM-Dextran(CM-DX-MP), UCON-3250-MP, UCON-2000-MP, Polyethylene Glycol(PEG-MP), EOPO-MP and Jeffamine-MP.

3.10 Morphology and grain size distribution of magnetic particles

The morphology observation of particle using transmission electron microscopy shows formation of larger agglomerates when the particles were coated with polymers.It is observed that diameter of each individual magnetic core is around 10 nm. This might be explained by the non-covalent interactions between the coating biopolymers of neighbour nanoparticles. According to figure-3.7 it is possible to conclude that the supports synthesized are not uniform in size and present a high polydispersitivity.

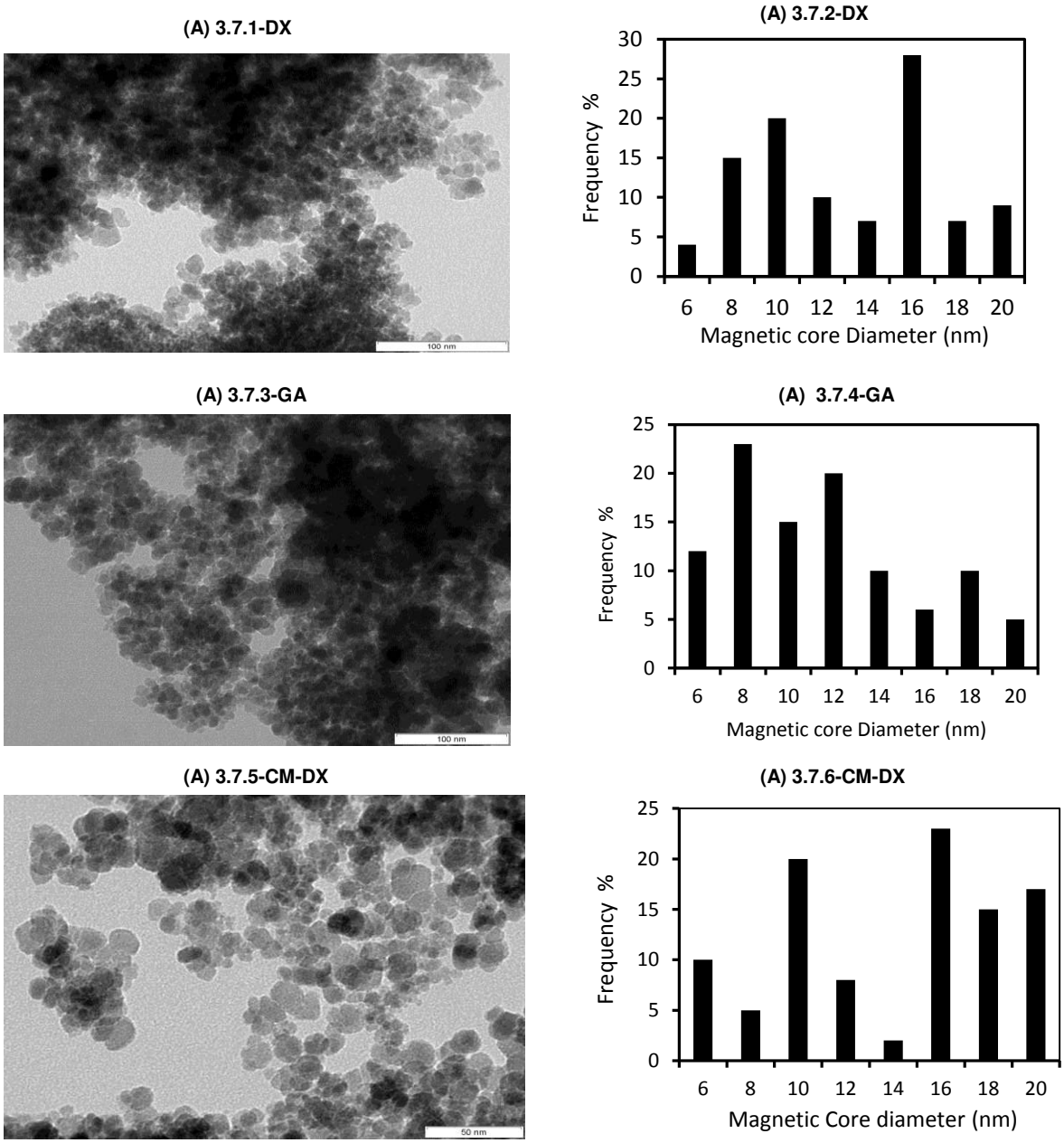

Figure 3.7 (A) Images and Grain size distribution from transmission electron microscope (TEM) for magnetic particles as Dextran (DX-3.7.1 & 3.7.2), GumArabic (3.7.3 & 3.7.4), CM-Dextran (3.7.5 & 3.7.6).

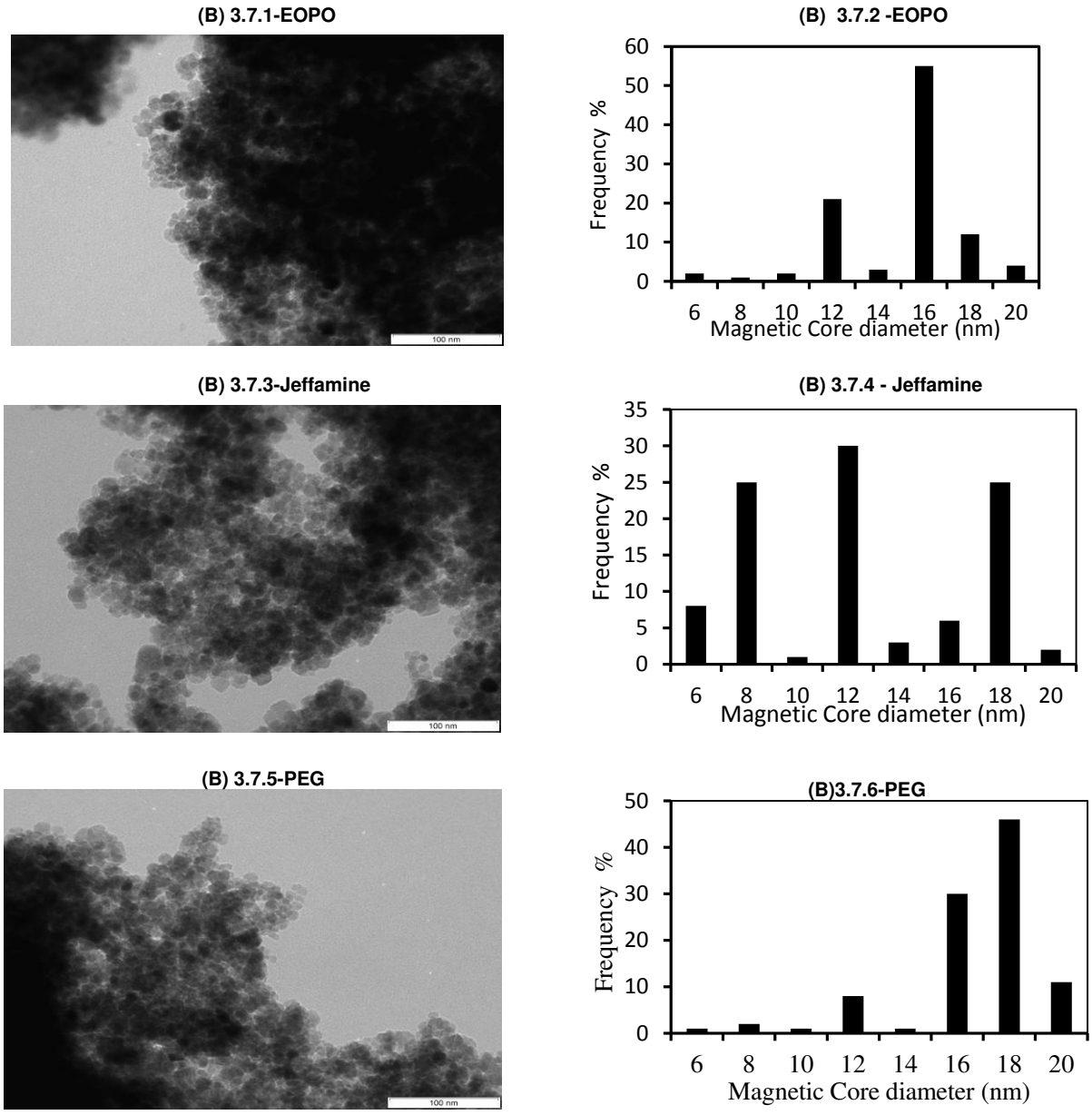

Figure 3.7 (B) Images and Grain size distribution from transmission electron microscope (TEM) for magnetic particles as EOPO-MP(3.7.1 & 3.7.2), Jeffamine-MP(3.7.3 & 3.7.4), Polyethylene Glycol(3.7.5 & 3.7.6), UCON-3250-MP(3.7.7 & 3.7.8).

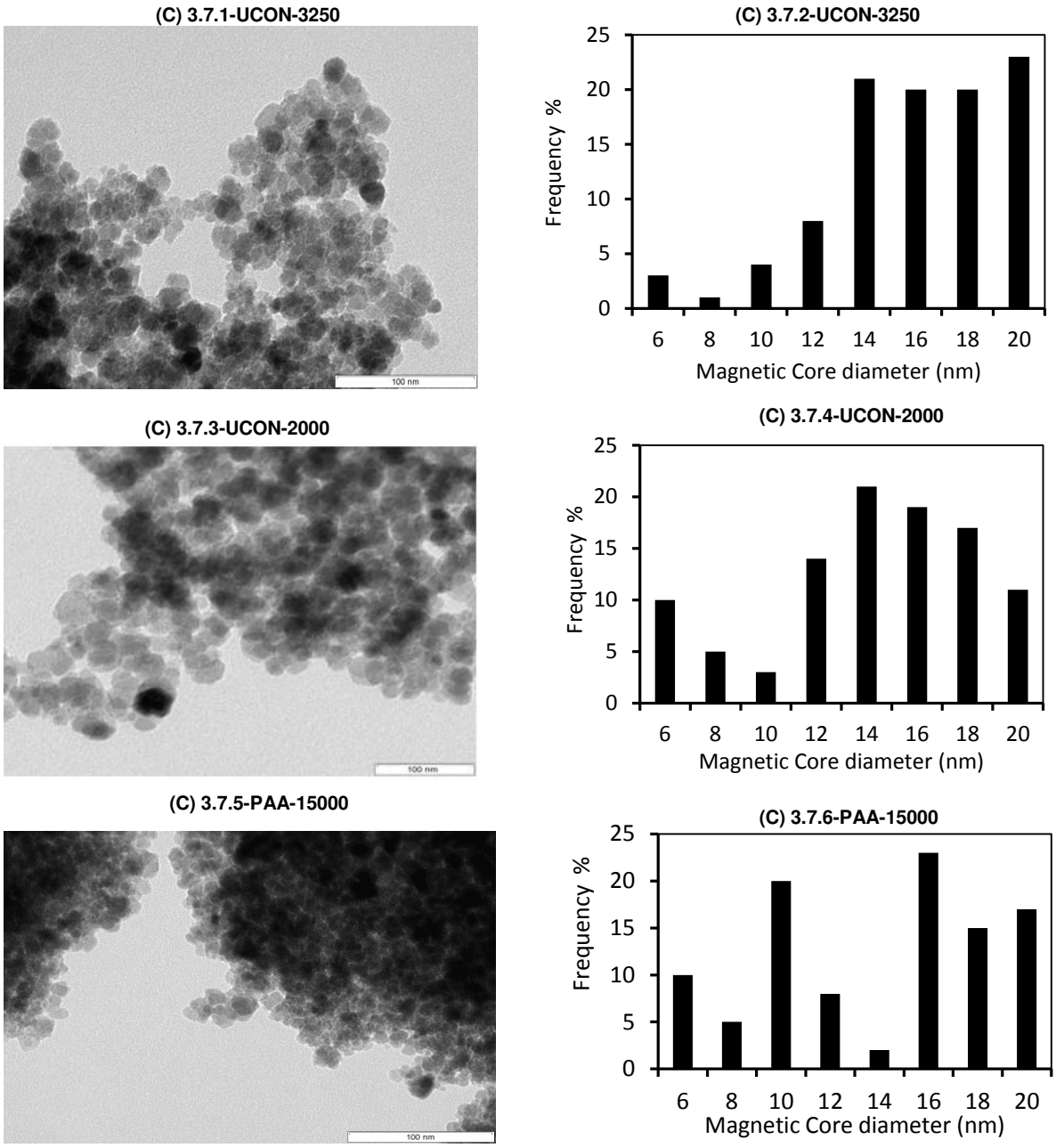

Figure 3.7 (C) Images and Grain size distribution from transmission electron microscope (TEM) for magnetic particles as UCON-3250-MP(3.7.1 & 3.7.2), UCON-2000-MP(3.7.3 & 3.7.4) and Polyacrylamide(3.7.5 & 3.7.6).

3.11 Charge of magnetic particles and effect of pH on zeta potential

Through result data as shown in Figure-3.8 it was observed that zeta potential values of all particle varies between -1 to -20 with variation of pH values between 3 to 12.Dextran coated MP have a zeta potential in the range of -4 to -18 which varies gradually with change of pH. At lower pH of 3 zeta potential value was -3.2 mV which increases gradually until pH 12 at which zeta potential value observed was -16.3. Highest zeta potential value observed at pH 11 for CM-Dextran coated MPs having value -19.5 which shows decrease at pH 12. In case of jeffamine modified particle initial zeta potential observed at pH 3 is -0.9 which increases gradually same as like biopolymer coated mp for higher values of pH. The value of zeta potential was observed maximum at pH 11 which is -6.3 which again decreases to -5 at pH 11.Particles coated with UCON-3520 also shows similar zeta potential decline trend as jeffamine having value of -7.1 at pH 12. Usually,the values of zeta potential are attributed to the oxygen groups available in the tetrahedrally and octahedrally structure. When particles are coated with the different polymers the global charge is altered according to the type of biopolymer used.

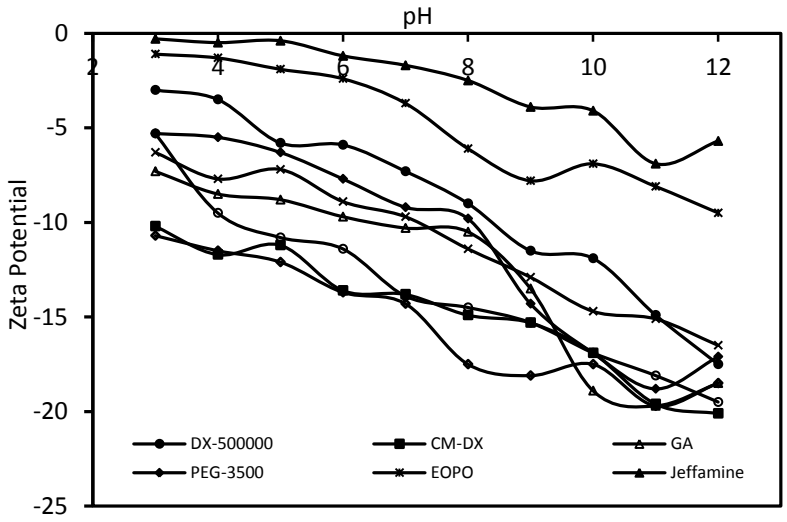

Figure. 3.8 Magnetic particles Zeta potential change with change of pH for Dextran(DX-500000),GumArabic (GA-MP), CM-Dextran (CM-DX-MP), UCON-3250-MP, UCON-2000-MP, Polyethylene Glycol(PEG-MP), EOPO-MP and Jeffamine-MP.

3.12 Selection of polymer-coated magnetic particle suitable for ATPS

MPs are commonly coated with biological and synthetic polymers with the aim of isolating the magnetic core against harsh conditions, improving biocompatibility, introducing functional groups for further chemical modification, and increasing colloidal stability. The polymers chosen in this work for the coating of MPs were Dextran-500000, Gum arabic, CM-Dextran, PEG-3500, EOPO, Jeffamine-M-2070, Polyacrylic acid sodium salt-15000, UCON-2000 and UCON-3520. This selection was based on commonly employed polymers in ATPS for antibody purification (e.g. PEG, Dextran, EOPO, UCON and

Jeffamine [10], as well as polymers previously selected for the coating of MP applied in antibody magnetic fishing.

In order to develop an hybrid process combining magnetic particles and aqueous-two phase extraction for antibody purification, the first step was the selection of the most suitable polymer coated MPs when employing typical ATPSs optimised for antibody separation[11].For this purpose, MPs coated with the selected natural and synthetic polymers with distinct properties, were prepared and then supplemented to PEG/Dextran ATPSs, and the partitioning of pure human IgG was investigated (figure 1 in SI) at different salt concentrations (100, 200, 300, 400 and 500 mM NaCl). In general it was observed that by increasing the salt concentration there is a decrease in the concentration of IgG in the lower dextran phase and a simultaneous increase in the concentration of IgG in the upper PEG-rich phase, particularly for salt concentrations greater than 200 mM. A rise in salt concentration is responsible for a decrease in the electrostatic potential difference between the upper and lower phases due to an even distribution of chloride ions between both phases. The rise of salt concentration also decreases non-specific ionic interactions, and therefore there is rise of hydrophobic interactions which promote the transfer of IgG from the dextran to the PEG phase. These favourable electrostatic interactions also present in upper phase between IgG and chloride ions. On the other hand, the amount of IgG adsorbed onto polymer coated MPs (usually stays at bottom phase) tend to increase with the increase in salt concentration (figure 3.9).

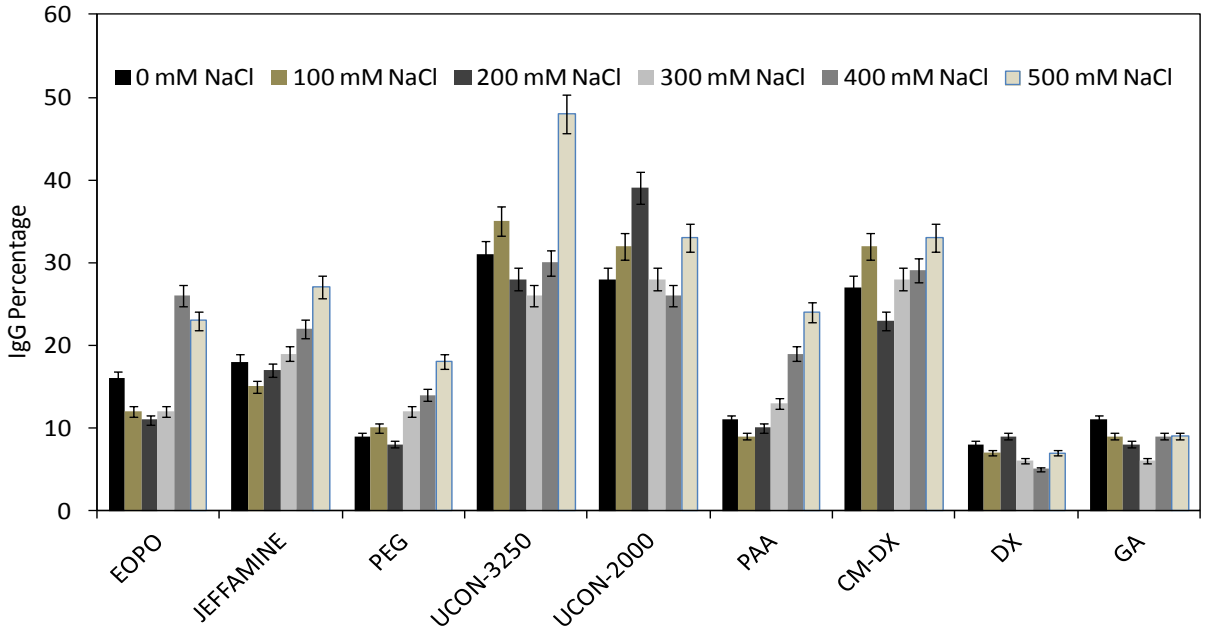

Figure. 3.9 Percentage of human IgG bound to MPs coated with natural and synthetic polymers in an ATPS system composed by PEG/Dextran for increasing NaCl concentrations (n=2).

This effect is particularly evident for EOPO, PEG, Jeffamine and PAA coated MPs. It is expected that the interactions between the antibody and the polymer coated particles are non-specific, hydrophobic and ionic in nature. Particles coated with UCON polymers and CM-dextran yielded the higher amounts of bound human IgG, for all salt concentrations tested, as opposed to the dextran and gum arabic coated MPs which bound the lower amounts of human IgG. This confirmed that these polymer coated particles were the most suitable to pursue with further surface modification through the introduction of specific antibody-binding ligands. It has been previously observed that dextran and gum Arabic coated magnetic particles formed a suitable platform for the affinity magnetic fishing of antibodies from purified and unpurified samples [12].

The interactions between the antibody and the Gum arabic as well as Dextran coated particles are non-specific, hydrophobic and ionic. Addition of salt is responsible for stronger interactions between magnetic particles and IgG whereas an ionic interaction becomes weak. Figure 3.10 (A) and 3.10 (C) shows the logarithum of the pure IgG partition coefficient in systems containing Gum arabic and Dextran coated MPs , respectively with increasing NaCl concentration.(For more information regarding partition coefficients of remaining all particles studied in this article please refer SI.figure 2. When NaCl was added in the system partition coefficient increases sharply and maximum IgG can be recovered in the upper phase. However, the concentration of IgG in the upper phase is about two orders of magnitude higher than the concentration in the lower phase for all higher range of salt concentrations studied. The presence of salt is responsible for high ionic strength when salt was added ionic strength increases to even higher level [13]. This increase in ionic strength is responsible for removal of bound water and exposure of the hydrophobic zones of the protein surface. However , as the concentration of NaCl increases, the electrostatic potential difference between the upper and lower phase will decrease proportionally as the chloride ions have an even distribution between both phases [14]. Hence, a favourable electrostatic interaction will also be present in the upper phase, but between the positively charge IgG and the negatively charged chloride ions.

3.13 Pure Protein Binding results of APBA modified magnetic supports having gum Arabic and dextran basic core in Dextran / PEG ATPS

The amino phenyl boronic acid modified magnetic nanoparticles having basic core coated with Gum arabic and dextran were tested for binding to pure solutions of human IgG. The buffer conditions used for binding human IgG on the magnetic supports were 20 mM HEPES and 1.5 M Tris-HCl. In terms of the elution conditions, the MPs were tested for lower pH elutions of upto pH 3 but in that case it was observed that purity was not that much efficient as like that obtain at pH 8.5. During this work, the elution at basic pH was selected as a result of separation and elution abilities. During the assessment for binding human IgG the bound and eluted antibody was monitored by BCA method. The results are shown in figure 3.10 (E & F). The partition coefficients results were better for GA-APBA MPs which was around 0.8 (figure 3.10-B) at salt concentration of 200 mM, on other hand for DX-APBA MPs (figure 3.10-D)

partition coefficient was 0.3. From binding and elution results also it was observed that GA-APBA modified MPs were more superior than DX-APBA MPs and hence only GA-APBA modified MPs were used for further studies.

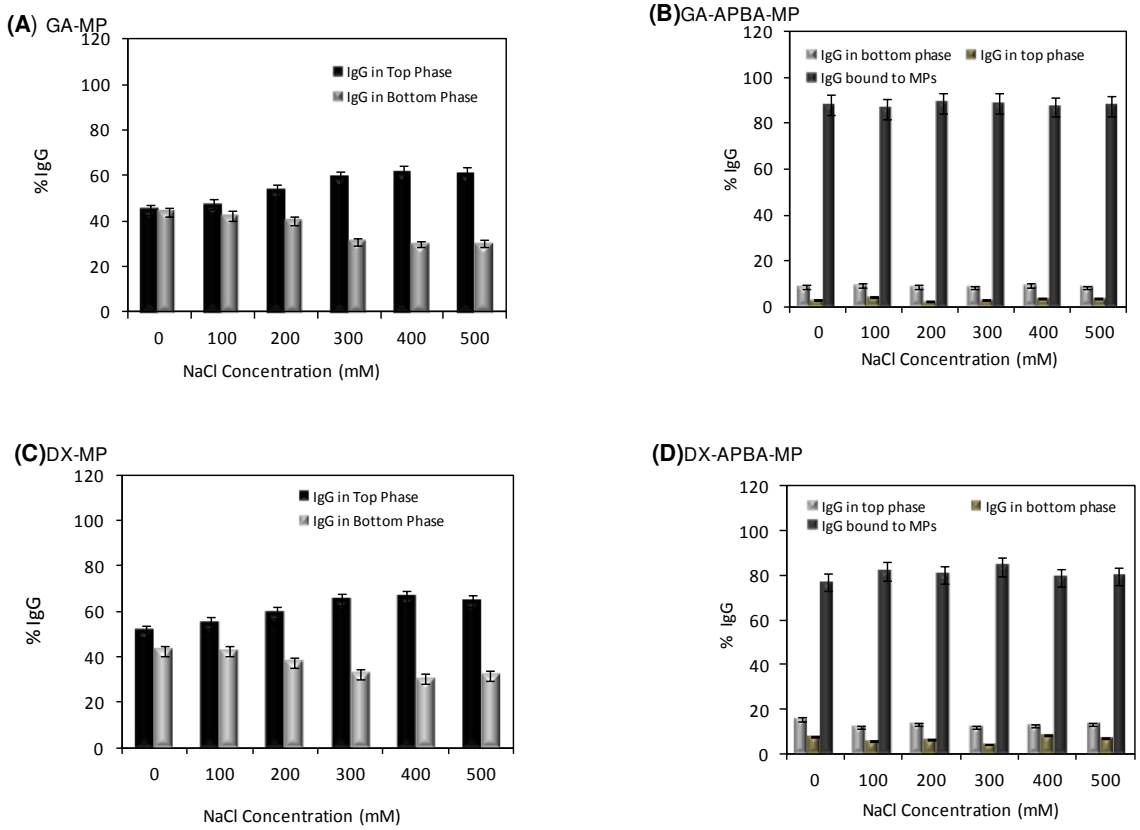

Figure. 3.10 Partition of pure human IgG in hybrid PEG/dextran systems supplemented with various magnetic particles at increasing salt concentration. (A) Gum arabic coated magnetic paricles (GA-MP), (B) Boronic acid modified magnetic particles having gum arabic polymer coated core (GA-APBA-MP) , (C) Dextran coated magnetic particles (DX-MP), (D) Boronic acid modified particles having dextran polymer coated magnetic core (DX-APBA-MP).

3.14 Elution Studies

IgG elution studies from amino phenyl boronic acid particles were performed by testing different eluents in particles into which IgG was previously adsorbed. It is difficult to recover IgG efficiently from boronic acid particles under acidic conditions. Maximum yield obtain from the mp support using citrate buffer at pH 3 is around 25%. APBA mnp shows excellent elution properties at pH 8.5. By increasing the concentration of Tris to up to 1.5 M the recovery yield could be improved to 95%. Small amount of elutions are observed by incubation with binding buffers. This can be due to weak non-specific interactions with the silica based support or to protein-protein interactions present as a result of multi-layer adsorption. The elution studies results were shown in figure 3.11 (A and B).

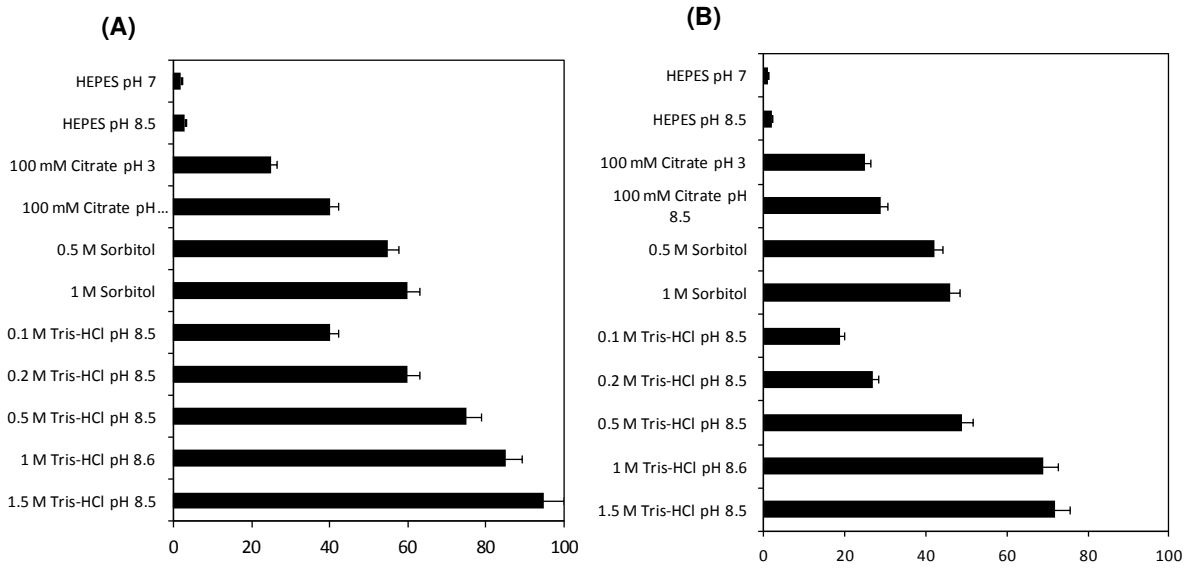

Figure. 3.11 Percentage of hIgG eluted from magnetic particles using various buffers at different pH conditions. (A) Boronic acid modified magnetic particles having gum arabic polymer coated core (GA-APBA-MP), (B) Boronic acid modified particles having dextran polymer coated m agnetic core (DX-APBA-MP).

3.15 CHO Cell supernant purification results using the hybrid system MATPS

The partition of IgG and the protein contaminants from a CHO cell supernatant in PEG / Dextran ATPSs supplemented with aminophenyl boronic acid modified MNPs having basic core coated with Gum Arabic MNPs was evaluated. In order to increase the IgG titre in the supernatant, which is considerably low (295 mg/L), and make the IgG quantification by chromatography easier and more accurate, it was added with pure IgG (from Gammanorm) to the supernatant to a final concentration of 1235 mg IgG/L. In this assays the spiked supernatant loading was of 20% (w/w).

According to the results obtained as shown in figure 3.12 (A) higher yields of IgG in the upper phase are obtained using high concentrations of NaCl. As the NaCl concentration has the most prominent effect in both yield and purity, this variable was studied for five different concentrations, namely 0, 100, 200,300,400 and 500 mM of NaCl concentrations. As expected, by increasing the concentration of NaCl, there is a decrease in the concentration of IgG in the lower dextran phase and a concomitant increase in the concentration of IgG in the upper PEG-rich phase. figure 3.12 (B) compares the IgG partition coefficients for different NaCl concentrations studied.The purity of both upper and lower phases as well as elutions from magnetic nanoparticles were analysed by SDS–PAGE (figure 3.13).

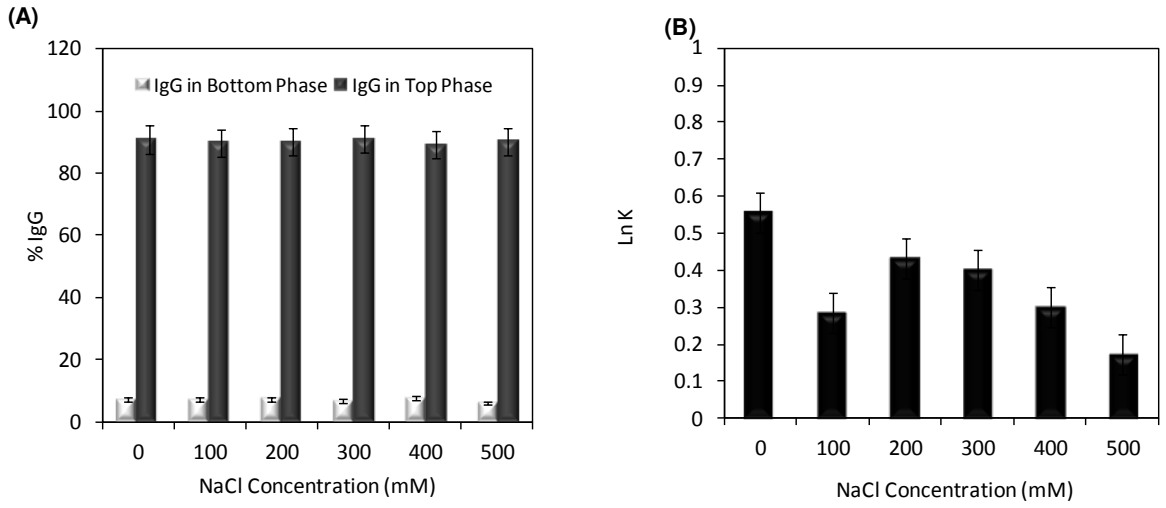

Figure. 3.12 (A) Percentage of IgG in crude samples present in the PEG/Dextran system and bound to GA-APBA-MP for increasing NaCl concentrations.**(B)** Logarithm of the crude IgG partition coefficient in systems containing GA-APBA-MP for increasing NaCl concentration.

After confirmation by SDS-PAGE electrophoresis of the binding and elution capacity of the support on the crude extract used, the samples collected were quantified by Protein-A HPLC in order to determine the amount of protein bound to and eluted from the support. From the HPLC results it was possible to predict that about 92 % of total IgG is eluted from the support at 200 mM salt concentration having purity level more than 98 % .

Table 3.1 compares the results obtained by our hybrid technology with magnetic fishing and ATPE alone. In magnetic fishing there is a direct contact between the GA-APBA-MP and crude samples containing antibodies – the yield of recovery was calculated as 67% with 74% purity. The binding and elution buffers employed were same as those used in the hybrid system. The benefits of direct magnetic fishing were associated with fast operation (only 15 minutes were required for incubation and there is no phase separation period), and the economy in reagents as no polymers are needed. Still, the purity achieved was quite low. On the other hand, ATPE using the same PEG-Dextran system but without magnetic particles provides only 23% yield with 39% purity. The time required for phase separation was 40 minutes whereas in the hybrid system the magnetic particles accelerated ATPS, as phase separation was reduced to 25 minutes (~35% reduction). One benefit when using ATPS alone is associated with buffers savings, as there is no need to wash the particles and elute the protein. The ATPS technique described in [18] was impressive even though purity that obtain was 80 % with almost all IgG recovery.

Table 3.1. Comparison of the hybrid process described in this work with direct magnetic fishing, ATPS and work from ref [13] and [20].

	Yield (%)	Purity (%)	Amount of MPs needed per ml of crude	Time required for phase separation in ATPE (min)	Time needed for incubation (min)	Ligand attached to MPs	Mg of Salt per gm ATPE	Eluent Buffer
Direct magnetic fishing	67	74	10 mg	NA	15	Boronic Acid	NA	Tris-HCl (pH-8.5)
ATPS alone [7]	23	39	NA	40	NA	NA	150 mg	NA
Hybrid process	92	98	5 mg	25	10-30	Boronic acid	11.6 mg	Tris-HCl (pH-8.5)
Process described in [13]	39	49	40 mg	NA	60	IgG	150 mg	3.5 M KSCN
Process described in [20]	< 98	80	NA	NA	NA	NA	600 mg	NA

In Table 3.1 it is also possible to benchmark our present technology with other similar reports on magnetic aqueous two-phase systems, as the possibility to combine magnetic particles with ATPS has been previously addressed. The article published in [19, 20] put forward a technique of accelerated phase separation using non-functionalised magnetic particles. Particles as such did not bind any solutes or materials on their surface. In a further publication [21] authors employed magnetic particles functionalized with an antibody (an extremely costly option) to enhance ATPS achieving a very poor purity level (49%) from crude samples, by using 3.5M KSCN solution as an eluent which represents an extremely harsh condition. The authors could not employ low pH for elution of the protein due to the dissolution of particles. In addition, in this work a high amount of particles were needed to achieve a very low purification level (40 mg of particles for 8 g of aqueous two-phase system; ratio of 5), with the limitation of partitioning the magnetic beads to the top phase, after one hour required for stirring all components of the mixture. Also, the amount of salt needed was high. Later, other authors [22] has disclosed the invention in

which a surfactant was added to an ATPS together with magnetic particles which makes the process more complex and expensive and generating difficulty in removal of surfactant from final product, generating purity constraint in end use of product [23]. Overall, the hybrid technology presents in this work employs a low amount of particles, produced at in a very inexpensive manner as no biological affinity ligands are used, and operates at extremely mild conditions, still reducing the time needed for incubation and phase separation while improving purity and yield.

3.16. Regeneration and antibody adsorption time Study

The particles show superior regeneration and reuse capacity. MPs can easily be regenerated and reused for more than five times. As shown in figure – 4 (A) it is observed that until fifth stage of recycle particles retain about 78-84 % protein binding capacity by considering initial protein binding capacity as 100 %.

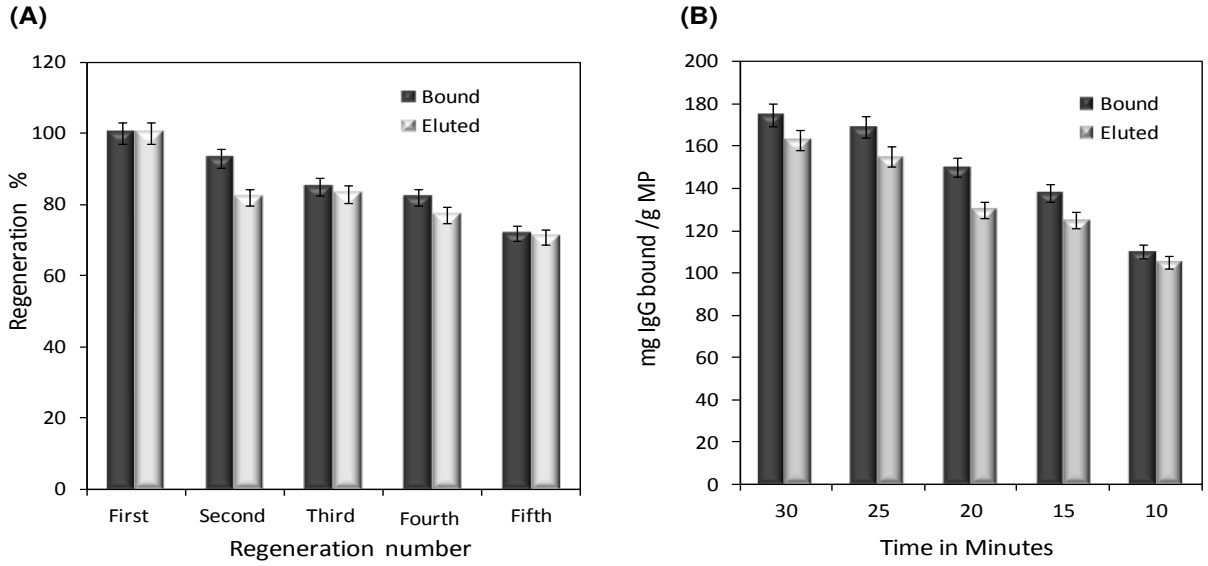

Figure. 3.13 (A) Regeneration percentage for GA-APBA-MP. (B) Quantity of IgG bound to the GA-APBA-MP supports at various time periods.

Other study performed with MP_Ga modified with amino phenyl boronic acid time bound test in order to know minimum time particles require to adsorb desired protein on its surface. This test found to be important while considering large scale application of particles for IgG purification. As shown in figure-3.13 (B) it was observed that minimum time required to particles for adsorbing maximum amount of IgG on its surface was 25 minutes in ATPS system of PEG/Dextran. Considerable adsorption was found to be in the test of 20 minutes but it will lead to loss of desirable protein and hence 25 minute incubation time is more preferred while utilizing the particles at large scale operation of ATPS coupled with High Gravity Magnetic Separation.

3.17. Magnetic particles characterization results of selected MPs having APBA

The coating of the particles with different biopolymers leads to an increase of the hydrodynamic diameter of the particles. The formation of larger agglomerates when the particles were coated has already been observed in other works [15]. It is observed that diameter of each individual magnetic core is around 10 nm. This might be explained by the non-covalent interactions between the coating biopolymers of neighbour MPs [16]. For the Gum Arabic Coated MPs, the average hydrodynamic diameter is around 650 nm and here it is also possible to conclude the presence of some agglomeration of the particles.

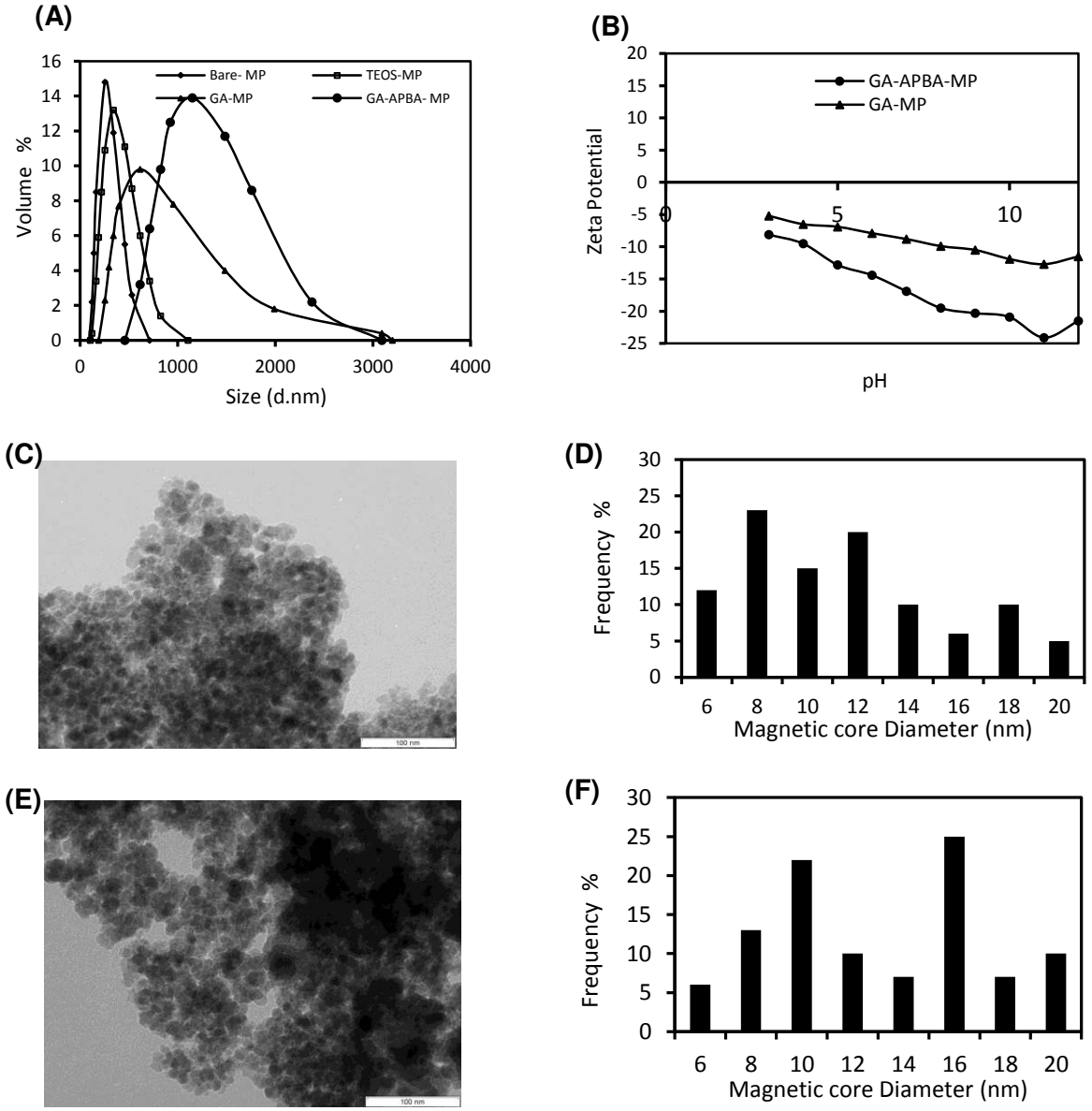

Figure 6. Magnetic particles characterization by DLS for particle size distribution of Bare-MP, TEOS-MP,GA-MP and GA-APBA-MP (A); Zeta potential change of GA-MP and GA-APBA-MP with change of

pH (B); TEM image of GA-APBA-MP (C) ; grain size distribution from TEM for GA-APBA-MP (D);TEM image of GA-MP (E); grain size distribution from TEM for GA-MP (F).

According to figure 3.14 (A) it is possible to conclude that the supports synthesized are not uniform in size and present a high polydispersitivity. The average hydrodynamic diameter for bare mp varies in the range of 240 nm. In case of particles coated with tetraethoxy silane rise in average hydrodynamic diameter observed to 310 nm. The final mp with aminophenyl boronic acid coating have average diameter in the range of 940-950 nm, eventhough some particles of size upto 3000 nm are also observed in the suspension.DLS results, shows that the average hydrodynamic diameter for PEG Coated MNPs, is around 585 nm. The mps with dextran-500000 have average diameter in the range of 550-575 nm, eventhough some particles of size upto 3000 nm are also observed in the suspension.UCON-3250 coated MP shows higher particle size in the range of 1650-1700 nm. Particles of CM-Dextran and UCON-2000 are of quite similar particle size range. EOPO coated magnetic particles also have higher range of particle size around 1000-1050 nm.

The majority of the particles dispersed in an aqueous solution will have a charged surface either by ionization of surface groups or adsorption of charged species [17]. This will modify the distribution and will increase the concentration of the surrounding ions. The liquid layer surrounding the particle exists as two parts; an inner region (Stern layer) where the ions are strongly bound and an outer (Diffuse layer) region where they are less firmly connected [18]. Within the diffuse layer there is a notional boundary inside which the ions and particles form a stable entity. When a particle moves (e.g. due to the voltage that is applied), ions within the boundary move it. Those ions beyond the boundary stay with the bulk dispersant [19]. This boundary which is usually called as slipping plane has a potential which is measured using DLS as zeta potential. The magnitude of the zeta potential gives an indication of the potential stability of the colloidal system [12]. Particles in suspension with a high zeta potential of the same charge tend to repel each other and resist aggregation. However, if the particles have low zeta potential values then there will be no force to prevent the particles coming together and flocculating [20]. It is conventionally assumed that high zeta potential values are below -30 mV or above +30 mV. This means that particles with zeta potentials more positive than +30 mV or more negative than -30 mV are normally considered stable. Through result data as shown in figure- 3.14 (B), it is observed that Gum Arabic coated MPs have a zeta potential in the range of -8 to -18 which varies gradually with change of pH. At lower pH of 3 zeta potential value was -7.3 mV which increases gradually until pH 12 at which zeta potential value observed was -18.5. Highest zeta potential value observed at pH 11 having value -19.7 which shows decrease at pH 12. In case of aminophenyl boronic acid modified particle initial zeta potential observed at pH 3 is -9.3 which increases gradually same as like Gum Arabic coated mp for higher values of pH. The value of zeta potential was observed maximum at pH 10 which is -22.9. The zeta potential decreases to -20.5 at pH 12.The values of zeta potential are attributed to the oxygen groups available in the tetrahedrally and octahedral structure. When particles are coated with the different

polymers the global charge is altered according to the type of biopolymer used [21]. The negative zeta potential of the Gum Arabic is mainly attributed to the carboxylic groups available on its structure.

With the VSM analysis it was possible to investigate the magnetic properties of the supports prepared. The curves represented on figure- 3.14 (G) presented a reversibility and symmetry which represents a typical no hysteresis curve characteristic of the superparamagnetic behavior of the particles synthesized.

TEM analysis confirmed the existence of magnetic cores in spherical shapes (figure 3.14 – C-F) with variations in diameter of magnetic core, some heterogeneous population is also observed since the size distribution is between 6 – 20 nm.

3.18 Conclusion

This work shows the applicability of using integrated process technology consisting of high gravity magnetic separation and aqueous two-phase extraction for the purification of human antibodies from CHO cell culture supernatant. The component that plays role in the extraction yield and purity of IgG for mnp adsorption as well as increasing concentration in the upper phase was the concentration of NaCl. The ATPS composed of 8% PEG, 5% Dextran and 200 mM NaCl afforded high recovery yield of IgG using MPs coated with aminophenyl boronic acid. This Hybrid process successfully used to purify monoclonal antibodies from a concentrated CHO cell culture supernatant, with a total yield of above 92 %, having purity of more than 98 % . These results provide excellent scope for large scale application of ATPSs coupled with magnetic particles for purification of therapeutic monoclonal antibodies.

References:

1. Adriaenssens E.M., L. S. M., Vandersteegen K., (2012). CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. Virology, 434(2), 265-270.
2. Aguilar O. et. al. (2010). Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soyabean proteins. Sep. Sci. Technol., 45, 2210-2225.
3. An X., S. Z., Zeng H.,. (2003). Preparation of highly magnetic chitosan particles and their use for affinity purification of enzymes. Journal of Chemical Technology & Biotechnology, 78(5), 596-600.
4. Asenjo J.A. and Andrews B.A. (2011). Aqueous two-phase systems for protein separation: a perspective. J. Chromatogr. A, 1218(49), 8826-8835.
5. Azevedo A.M. et al. (2009). Affinity-enhanced purification of human antibodies by aqueous two-phase extraction. Sep. Purif. Technol., 65, 31-39.
6. Azevedo A.M. et. al., & (2009). Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography. J. Chromatogr. B, 877(50 - 58).
7. Bandeira V., P. C., Rodrigues A.F., (2012). Downstream processing of lentiviral vectors: releasing bottlenecks. Hum. Gene Ther. Method, 23(4), 255-263.
8. Barbosa H.S.C., H. A. V., Brocchini S., Slater N.K.H., Marcos J.C. (2010). Dual affinity method for plasmid DNA purification in aqueous two-phase systems. J. Chromatogr. A, 1217(9), 1429-1436.
9. Bensch M. et. al. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62, 2011-2021.
10. Bensch M., S. B., Hubbuch J,. (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.
11. Bolognese B., N. B., Pico G.,(2005). Application of the aqueous two-phase systems of ethylene and propylene oxide copolymer-maltodextrin for protein purification. J. Chromatogr B, 814, 347-353.
12. Bolton G.R. et. al. (2011). Addressing the challenges in downstream processing today and tomorrow. Biopharm Int., 24, S8-S15.
13. Chen J, T. J., Zhang Y, Wasserman A, Conley G, DiLeo M, Haimes E, Nixon AE, Ley A. (2010). The distinctive separation attributes of mixed-mode resins and their applications in monoclonal antibody downstream purification process. J Chromatogr A, 1217, 216-224
14. Chen S. et. al. (2009). A screening paradigm for the design of improved polymer-coated superparamagnetic iron oxide nanoparticles. . Journal of Materials Chemistry, 19(35).
15. Chusainow J. (2009). A study of monoclonal antibody producing CHO cell lines - what makes a stable high producer? Biotechnol. Bioeng.,102, 1182-1196.

16. Ditsch A. et. al. (2005). High-Gradient Magnetic Separation of Magnetic Nanoclusters. Industrial & Engineering Chemistry Research, 44(17), 6824-6836.
17. Faria J. T., S. F. C., Converti A.,Passos F. M. L.,Minim V. P. R.,Minim L. A., . (2009). J. Chromatogr., B 877, 3031-3037
18. Frakas T., S. H., Tjerneld F.,. (1996a). Partitioning of bmannanase and a-galactosidase from Aspergillus niger in Ucon/Reppal aqueous two phase system. Bioseparation, 6, 147-157.
19. Gagnon P. (2008). The emerging generation of chromatography tools for virus purification. BioProcess Int, 6(S6), 24-30.
20. Gao J. et. al. (2012). Antibody affinity purification using metallic nickel particles. Journal of Chromatography B, 895-896, 89-93.
21. Gerster P., K. E. M., Hammerschmidt N.,. (2013). Purification of infective baculoviruses by monoliths. J. Chromatogr. A, 1290, 36-45.
22. Goloverda G. et. al. (2009). Synthesis of ultrasmall magnetic iron oxide nanoparticles and study of their colloid and surface chemistry. . Journal of Magnetism and Magnetic Materials, 321(10), 1372-1376.
23. Guan Y., L. T. H., Treffry T.E., Zhou C.L.,Wilkinson P.B., . (1996). Enzyme Microb. Technol., 19(6), 446.
24. Guoqiang D., K. R., Mattiasson B.,. (1994). Integration of aqueous two-phase extraction and affinity precipitation for the purification of lactate dehydrogenase. J Chromatogr A, 668, 145-152.
25. Heebol-Nielsen A., J. S., Hobley TJ.,Thomas ORT.,(2004). Superparamagnetic cation-exchange adsorbents for bioproducts recovery from crude process liquors by high gradient magnetic fishing. Sep Sci Technol, 39, 2891-2914.
26. Holschuh K. and Schwammle A. (2005). Preparative purification of antibodies with protein A – an alternative to conventional chromatography. J Magn Magn Mater, 293, 345-348.
27. Horák D. et. al. (2007). Preparation and properties of magnetic nano- and microsized particles for biological and environmental separations. Journal of Separation Science, 30(11), 1751-1772.
28. Hubbuch J.J. and Thomas O.R.T. (2002). High gradient affinity separation of trypsin from porcine pancreatin. Biotechnol Bioeng, 79, 301-313.
29. Jain E, a. K. A. (2008). Upstream processes in antibody production - evaluation of critical parameters. Biotechnol. Adv., 26, 46-72.
30. Jefferis R. (2009). Glycosylation as a strategy to improve antibodybased therapeutics. Nature, 8, 226-234.
31. Jin M. et. al. (2010). Profiling of host cell proteins by two dimensional difference gel electrophoresis (2D-DIGE) - implications for downstream process development. Biotechnol. Bioeng., 105, 306-316.

32. Jonsson M. and Johansson H.O. (2003). Protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer. J Chromatogr A 983, 133-144.
33. Jungbauer A. and Peng J. (2011). Continous bioprocessing: an interview with Konstantin Konstantinov from Genzyme. Biotechnology J.,6(12), 1431-1433.
34. Kandula S. et. al. (2009). Design of a filter train for precipitate removal in mAb downstream processing. Biotechnol. Appl. Biochem., 54, 149- 155.
35. Kang S. et. al. (2009). Bioconjugation of poly (ethylene glycol) methacrylate)-coated iron oxide magnetic nanoparticles for magnetic capture of target proteins. Macromolecular Research, 17(4), 259-264.
36. Kepka C., C. E., Persson J., Stahl A., Lagerstedt T., Tjerneld F., Veide A., (2003). Extraction of an intracellular recombinant cutinase from E. Coli cell homogenate using thermoseparating aqueous two phase system. Journal of Biotechnology, 103 165-181.
37. Kramberger P., H. R. C., Herman R.E., Smrekar F., Peterka M.(2010). Purification of the Staphylococcus aureus bacteriophages VDX-10 on methacrylate monoliths. J. Virol. Methods, 166(1-2), 60-64.
38. Kuczewski M. et. al. (2011). A single-use purification process for the production of a monoclonal antibody produced in a PER.C6 human cell line. Biotechnology Journal, 6(1), 56-65.
39. Kumar A., W. P. O., Kepka C., Galaev I.Yu, Mattiasson B., (2003). Purification of histidine-tagged single chain Fvantibody fragments by metal chelate affinity precipitation using thermo-responsive copolymers. Biotechnol Bioeng, 84, 495-503.
40. Lacki K.M. (2012). High-throughput process development of chromatography steps: advantages and limitations of different formats used. Biotechnol. J., 7, 1192-1202.
41. Liu HF., M. J., Winter C., (2010). Recovery and purification process development for monoclonal antibodies. mAbs, 2, 480-499.
42. Liu, X., et al., 2004. Surface Modification and Characterization of Magnetic Polymer Nanospheres Prepared by Miniemulsion Polymerization. Langmuir, 20(23), 10278-10282.
43. Luechau F., L. T. C., Lyddiatt A.,(2011). Recovery of B19 virus-like particles by aqueous two-phase systems. Food Bioprod. Process, 89(4), 322-327.
44. Mattiasson B., K. A., Ivanov A.E., Galaev I.Yu.,. (2007). Metalchelate affinity precipitation of proteins using responsive polymers. Nat Protoc, 2, 213-220.
45. Mazumder J., Z. J., Ray AK.,. (2010). Optimal design of liquid-solid circulating fluidized bed for continuous protein recovery. . Powder Technol. , 199(1), 32-47.
46. Meyer A., H. D., Gomes CSG, Hobley TJ, Thomas ORT, Franzreb M,. (2005). Demonstration of a strategy for product purification by high gradient magnetic fishing. Biotechnol Prog, 21, 244-254.
47. Nfor B.K., V. P. D. E. M., Hubbuch J., Ottens M.,. (2009). Rational and systematic purification process development: the next generation. Trends Biotechnol, 27, 673-679.

48. Nishio K. et. al. (2008a). Development of novel magnetic nano-carriers for high-performance affinity purification. Colloids and Surfaces B: Biointerfaces, 64(2), 162-169.
49. Öztürk, N., et al.,2007.Silane-Modified Magnetic Beads: Application to Immunoglobulin G Separation Biotechnology Progress, 23(5), 1149-1156.
50. Pereira M., W. Y. T., Venaˆ ncio A., Teixeira J.,. (2003). Aqueous twophase extraction using thermoseparating polymer: a new system for the separation of endo-polygalacturonase. Biochem Eng J, 15, 131-138.

CHAPTER NO: 4

GREEN POLYMER FROM PRODUCTION TO APPLICATION IN BIOPROCESSING

SUMMARY

A fucose-containing extracellular polysaccharide (EPS) produced by *Enterobacter* A47 using glycerol as the carbon source, was employed as a coating material for magnetic particles which were subsequently functionalized with artificial ligand to capture antibodies. The magnetic particles performance in purification of antibody in magnetic separator as well as PEG-Dextran ATPE system was investigated. In direct magnetic separation using pure protein solutions of human IgG (hIgG) and bovine serum albumin (BSA), MP-EPS-22/8 bound 120 mg of IgG/g of particles, while binding only 10 ± 2 mg BSA/g MP. In magnetic particles accelerated ATPE system, particles exhibited good performance for partitioning of hIgG in desired phase as well as recovery by magnetic separation. The particles able to bind 145 mg of IgG/g of particles which is quite high as compared to direct magnetic separation. The theoretical maximum capacity was calculated as 410 ± 15 mg hIgG adsorbed/g MNP with a binding affinity constant of $4.3 \times 10^4\ M^{-1}$. In multiple extraction steps the particles were able to bound around 95 % of IgG loaded having purity level of 98.5%. The particles can easily be regenerated, recycle and reuse for five cycles with minor loss of capacity. The EPS polymer coating allowed both electrostatic and hydrophobic interactions with the antibody to enhance specificity for targeted products. This study will be highly useful for utilisation of EPS coated magnetic particles modified with artificial ligand for IgG purification.

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4.1. Introduction

The need for biological molecules as proteins, gene or viral vectors and cells in purified forms is usually a challenging and costly task [1]. Antibodies are an extremely important class of biopharmaceuticals as market reaches to 65 billion USD [2]. In this respect, chromatography is the most well established separation methods both at laboratorial and industrial settings. However, the impossibility of chromatographic methods to accompany increasing production titers, flexibility in production and the high-costs associated, are driving the search and re-evaluation of non-chromatographic technologies. Within these, aqueous two phase systems and magnetic fishing appear as potential candidates to be employed in combination or as a substitution of chromatographic steps [3]. Very recently, our groups presented a novel methodology which combined aqueous two phase extraction with magnetic fishing, named as a hybrid process, which yielded high yields of antibody recovery (92%) with high purity (98%) [4].

In order to employ magnetic particles in bioseparation processes, there is the need to coat the magnetic core In order to employ magnetic particles in bioseparation processes, there is the need to coat the magnetic core with either biological or synthetic polymers. The main role of magnetic particles coating is the protection of the core structure with a layer through encapsulation or through the establishment of strong electrostatic and hydrophobic interactions in order to isolate the core against harsh conditions. Polymer coating also improve biocompatibility of the particles and increase functionalization for further modifications [5-7]. The coatings most commonly used include: organic coatings such as surfactants [8, 9] and polymers or inorganic coatings as silica[10], carbon [11, 12] and precious metals [13, 14]. In the last years the coating of MNPs with polymers, particularly biopolymers such as polysaccharides [15], attracted attention of researchers to be known to increasing biocompatibility[16],chemical functionality and colloidal stability of nanomaterials [17]. In addition, biopolymers are renewable, non-toxic and biodegradable which make them an environmental and sustainable choice [18]. The combination of natural polymers with superparamagnetic particles (MPs) results in stable materials with low unspecific binding and available chemical groups for further ligand attachment, as shown for gum Arabic [19] and dextran [20] which were suitable for the creation of magnetic supports for bioseparation processes.

Production of polysaccharides by microbial fermentation has advantages in comparison to their extraction from other natural sources, since microorganisms usually exhibit higher growth rates and the manipulation of production conditions is much easier. Unlike other sources, microbial fermentation is not influenced by climate changes or seasonality [21].On the other hand, industrial microbial production is limited by the high cost of the most commonly used carbon sources (e.g. glucose, starch, sucrose and fructose) [22]. However, some studies revealed that it is possible to overcome this limitation by replacing those traditional substrates by low cost carbon sources like agro and industrial wastes or byproducts (e.g. glycerol byproduct) which contribute for the reduction of production costs [23]. The polysaccharides

produced by microorganisms can be divided into intracellular, structural and extracellular polysaccharides or exopolysaccharides (EPSs). Over the recent decades, several EPSs have been reported, and their composition, structure, biosynthesis and functional properties extensively studied [24]. EPSs are synthesized by the cells intracellular and exported to the extracellular environment as macromolecules. Furthermore, EPS extraction processes are easier being an advantage comparing to other natural polysaccharides [25].

EPSs are mainly composed of neutral sugars monomers, but acidic or amino-sugars are also common constituents. Although glucose and galactose are the common sugar residues in EPS structures, some polysaccharides have an increased value due to their content in certain rare sugars, which occur rarely in nature, such as fucose. Fucose-containing EPSs, in addition to their functional properties, including their rheological behavior in aqueous medium, thickening, emulsion forming and stabilizing capacity and flocculating activity, have increased market value, since fucose is one of the rare sugars, difficult to obtain, with many applications that include cosmetics and pharmaceuticals. Fucose-containing EPSs have been reported to be produced by several bacterial genera, including *Klebsiella*, *Clavibacter*, *Escherichia* and *Enterobacter* [26]. In this work, a fucose-containing EPS polymer having unique characteristics and developed by using glycerol as carbon source utilizing *Enterobacter* A47 (DSM 23139) used as a coating material for MNPs. EPS coated particles were then functionalized with an artificial ligand to capture antibodies through magnetic fishing and an hybrid process combining both ATPS and magnetic fishing.

4.2 Materials and Methods

4.2.1 Chemicals

Cyanuric Chloride, Tris(Hydroxymethyl)amino methane, (3-Aminopropyl) triethoxysilane (APTES), 3-hydroxyanilin, Ferric sulfatehydrate, Ferrous sulphate heptahydrate, HEPES, Anthrone, Sulfuric Acid, Absolute ethanol, Hydrochloric acid, Sodium Chloride, Sodium-di-hydrogen Phosphate 1-hydrate, (Di) Sodium-hydrogen Phosphate 2-hydrate, Sodium Hydroxide, were purchased from Sigma – Aldrich. Polyclonal human immunoglobulin G (IgG) for therapeutic administration (product name: Gammanorm) was purchased from Octapharma, as a 165 g/L solution containing 95% of IgG. 4-amino-1-naphtol hydrochloride, Poly (ethylene glycol) with molecular weight 3,350 and 8,000 was purchased from Sigma (St. Louis, MO, USA). Dextran with an average molecular weight of 500,000 was purchased from Fluka (Buchs, Switzerland). Glycine was purchased from Acros. Ninhydrin and Ammonium hydroxide were purchased from Fluka. Nitrogen was from Air Liquide.

4.2.2. METHODS

4.2.2.1 Biopolymer production

Enterobacter A47 (DSM 23139), previously isolated and stored at -20ºC was reactivated in a chromagar plate. The pre-inoculum for the bioreactor cultivation was prepared by growing a single colony isolated from the chromagar plate on medium LB (50mL) in shake flasks, for 48h at 30ºC, in an incubator

shaker (200 rpm). Afterwards, the inoculums bioreactor experiments was prepared by inoculating the pre-inoculum culture (20mL from LB medium) in slightly modified Medium E* (200mL, pH 7.0), supplemented with $MgSO_4$, mineral solution and commercial glycerol (40g/L) as the sole carbon source, as described previously [23], in an incubator shaker (200 rpm), for 48h at 30ºC. Finally, the inoculums for bioreactor were prepared by inoculating 80mL of pre-inoculum in Medium E* in 800mL of new Medium E* with the same composition distributed for 4 shake flasks (20mL of culture plus 200mL of Medium E* each), for more 72h at 30ºC and 200rpm.

The 5L bioreactor (BioStat B-plus, Sartorius) containing 2 L of Medium E*, supplemented with glycerol (at a concentration of ~25g/L), was inoculated with the culture (800mL). Afterwards, the bioreactor was operated as described in article [23]. Briefly, it was operated in a batch mode until, approximately, the end of the exponential phase of bacteria's growth curve and, in a fed-batch mode, for the next four days, by supplying the bioreactor with cultivation Medium E*, with a glycerol concentration of 200g/L, at a constant rate of 10mL/h. Temperature and pH were controlled at 30±0.1ºC and 7.00±0.05, respectively. The aeration rate (0.125vvm, volume of air per volume of reactor per minute) was kept constant throughout the cultivation, and the dissolved oxygen concentration (DO) was controlled by two 6-blade impellers. During the fed-batch phase, the DO was maintained below 10%. Culture broth samples were recovered over time in order to evaluate the bacteria's growth curve by measuring the absorbance at 450nm and also the culture broth viscosity was analyzed using a viscometer (Brookfield VD-II).

The culture broth recovered from the bioreactor at the end of the cultivation was diluted with deionized water (1:5, v/v) for viscosity reduction. The diluted culture broth was subjected to thermal treatment (70ºC, 1h) to inactivate bacterial enzymes that might cause polymer degradation during the subsequent purification steps. Afterwards, bacterial cells and any remaining denatured proteins were removed by centrifugation (8000rpm, 1h) and the cell-free supernatant was purified by diafiltration, concentrated and freeze dried.

4.2.2.2. Extracellular Polysaccharide (EPS) coating and functionalization of magnetic particles

Basic magnetic core synthesis of MPs was carried out as per protocol explain in [3]. The coating process was carried out using mechanical stirring of TEOS coated MPs (10 mg/ml) with solution of EPS polymer (0.3 gm dissolved in 50 ml milli-Q water). After mechanical stirring the particles were washed five times with distilled water and aminated using (3-Aminopropyl) triethoxysilane (APTES). Amination was followed by resuspension of particles in 50% (v/v) acetone: water mixture and reaction with 5 molar equivalents of cyanuric chloride based on the amount of amines available on the support. The reaction was carried out for 2 hours at 0°C having 300 rpm. In the end of this reaction the particles were washed one time with acetone, one time with 50% (v/v) acetone/water and finally five times with water. The first nucleophilic substitution reaction was carried out using 3-hydroxyaniline in water, by adding 2 equivalents relative to the amount of amines on the supports. The time span of reaction was 24 hours with stirring at 30°C. After the reaction the particles were washed five times with water. The second nucleophilic

substitution was carried out using 5 molar equivalents of 4-amino-1-naphtol-hydrochloride, dissolved in 50% (v/v) DMF/water. Reaction was carried out for 48 hours with stirring at 90°C. The particles were then washed one time with 50% (v/v) DMF/water and more five times with water. Finally, the particles modified with ligand 22/8 were resuspended in water.

4.2.2.3. Magnetic Aqueous Two-Phase Extraction

Aqueous two phase extraction system made of polyethylene glycol (3350) and Dextran (500,000) was used to investigate biopolymer coated MPs performance. For preparation of ATPE system PEG-3350 of 8 % (w/w) and Dextran-500000 of 5 % (w/w) were weighed in 15 mL graded ATPE glass tube. The magnetic particle concentration of 0.02 % (w/w) was added in each system and total weight of 5 gram was always kept constant. Pure IgG extraction studies were performed by adding 1 mL of 1 g/L IgG stock solution. In supernatant IgG extraction studies the supernant loading of the systems ranged from 1 to 1.5 ml of 1.35 g/L IgG containing solution. Total final weight of 5 g was balanced by adding water (Milli-Q). Salt concentration was varied between 100 mM to 500 mM for all systems. After adding all components through was carried out using a vortex shaker and then allowed for phase segregation for 2 to 4h at room temperature. Phase segregation was followed by positioning of test tubes on a magnetic separator for recovery of magnetic particles back from tube. Samples of each phase in system were collected. In order to quantify amount of IgG adsorbed by particles elution studies were afterwards performed in order to assess the amount of adsorbed IgG by MPs. Five subsequent MPs washes were made the first one with Milli-Q water and then four with 50 mM phosphate buffer of pH 8. Five times MNP elutions were made using 50 mM Glycine-NaOH buffer of pH 11. The quantity of IgG in both bottom and top phase followed by elution's from MPs were analysed for IgG quantification by HPLC.

4.2.2.4. Direct magnetic fishing of antibodies

The functionalized MP-EPS-22/8 were tested with a pure solution of hIgG, and with a pure solution of BSA. The particles suspensions were washed firstly with 0.1 M NaOH prepared in 30% (v/v) isopropanol, followed by washing with deionized water. After washing the nanoparticles suspensions were equilibrated with 50 mM phosphate buffer of pH 8. After washing and equilibration of support the supernatants were removed and added with 500 µl of a hIgG or BSA solution of 1 mg/ml concentration diluted in 50 mM phosphate buffer. The incubation was carried out for 15 minutes at room temperature with constant stirring. The supernatant was then collected and the particles were washed five times with 500 µL binding buffer. After preliminary washing steps elution of bound protein was then carried out using 50 mM Glycine – NaOH buffer of pH 11. All samples were analyzed by BCA assay with microplate reader in order to quantify the amount of protein bound to and eluted from the supports. In order to compare results the non-modified particles and ligand modified particles were tested at the same time and in the same conditions of the magnetic supports.

4.2.2.5. Partition equilibrium Studies

Partition equilibrium experiments were performed with solutions of human IgG having concentration in the range of 0-25 mg/ml and having quantity of 500 µl each. Dilution of IgG sample were carried out in 50 mM phosphate buffer of pH 8.The MPs concentrations were maintain at 10 mg/ml thought the experiment. For control MP-EPS were used in order to study relative adsorption. The samples were incubated for 12 h at room temperature, after which the supernatants were collected and the amount of free protein quantified by the BCA protein assay. Experimental data fitted with Scatchard plot and was represented by,

$$q = \frac{Qmax.Ceq}{Kd + Ceq}$$

In which q is the amount of bound protein in equilibrium per volume of solid support, Ceq is the concentration of bound protein in equilibrium, corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent) and the dissociation constant.

4.2.2.6. Regeneration and reuse of MP-EPS-22/8

MP-EPS-22/8 were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The 22/8 functionalized (MP-EPS-22/8) (500 µl with 10 mg/ml) were tested with 500 µl of each of the pure hIgG solution, by incubating for 15 minutes at room temperature. The liquid in which particles were suspended were removed by magnetic separation and then washed two times with 500 µl binding buffer (50 mM Phosphate, pH-8). After washing, MPs were eluted five times using 500 µL elution buffer (50 mM Glycine-NaOH, pH-11). All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

4.2.2.7. Studies of incubation time for direct as well as ATPE based separation process

Incubation time plays important role to adsorb antibody on the surface of magnetic particles. Functionalized MP-EPS-22/8 were studied in order to finalise exact incubation time to be provided to have optimum separation ability. This study was carried out separately for direct magnetic separation and aqueous two phase extraction (ATPE). For direct magnetic separation incubation time study five different test samples of MP-EPS-22/8 having 500 µl volume were incubated at room temperature with 500 µl hIgG solution (1 mg/ml) for different time intervals mainly 5,8,10,12,14,16,18 and 20 minutes respectively. Incubation was followed by washing the particles two times with 500 µl binding buffer (50 mM Phosphate of pH 8) and five times elution using elution buffer (50 mM Glycine-NaOH of pH 11), in order to know maximum hIgG that we able to elute. All washes were collected and quantified by BCA

method. In case of ATPE experiment binding time study five different ATPE systems were prepared having composition as explained in section 2.2.4. Incubation time was varied for each system and allowed for phase segregation. After phase segregation particles were separated and washed in the same manner as above to recover hIgG for quantification.

4.2.2.8 Crude IgG extracts purification

Magnetic supports MP-EPS-22/8 were tested with CHO cell culture supernatants in order to estimate efficiency of MPs for crude purification. For comparison of the results, testing of crude extract was carried out using both techniques i.e. direct magnetic separation as well as ATPE technique. In case of direct method, 500 µl MP-EPS and MP-EPS-22/8 with a concentration of 10 mg/ml were incubated with 500 µl of the crude extract respectively, without any pre-treatment, for 20 minutes at room temperature. For ATPE technique, the particles were incubated for 40 minutes at room temperature in ATPE system as described in section 2.2.4. After incubation the particles were separated and supernatants were collected. The separated particles were then washed five times with 500 µl binding buffer (50 mM Phosphate, pH 8). After washing, MPs were eluted using elution buffer (50 mM Glycine-NaOH of pH 11), in order to study the best elution conditions. All collected samples were quantified by affinity chromatography using Äkta purifier system from GE Healthcare (Uppsala, Sweden), using porous protein A affinity column from Applied Biosystems (Foster City, CA, USA). The BCA method and gel electrophoresis (12.5% Acrylamide/Bisacrylamide) in denaturing conditions by SDS-PAGE was also used for further analysis. The respective gels were prepared according to a standardized protocol. The low molecular weight marker and the samples for running gels were prepared by adding 2.5 and 10 µl of each sample, respectively, as well as 5 µl of sample buffer and boiled for two minutes immediately before applying to the gel. The gel was run for 80 minutes at 150 V and 250 mA by adding an electrophoresis Tris – Glycine Buffer (SDS-PAGE). For detection of the protein bands, the gel was stained with silver stain Biorad kit.

4.2.2.9. Analytical techniques

Culture broth samples recovered over time were centrifuged at 10,000 rpm for 10min, for cell separation. The cell-free supernatant was stored at -20ºC for posterior determination of glycerol and ammonium concentrations, and for the quantification of the EPS produced. The cell pellet was used for the gravimetric determination of the cell dry weight (CDW), after washing three times with deionized water (resuspension in water, centrifugation at 10,000rpm, for 10min, and, finally, resuspension in deionized water, filtration through 0.20µm filters, and dried in an oven at 100ºC for 24h).

Glycerol concentration in the cell-free supernatant was determined by high performance liquid chromatography (HPLC) with an Aminex HPX-87H column (BioRad), coupled to a refractometer. The analysis was performed at 50ºC, with sulphuric acid (H_2SO_4 0.01N) as eluent, at a flow rate of 0.6 mL/min. Ammonium concentration was determined using a potentiometric sensor (Thermo Electron Corporation, Orion 9512).Cell-free supernatant was dialyzed with a 10,000 MWCO membrane (SnakeSkin^{TM} Pleated Dialysis Tubing, Thermo Scientific), against deionized water (48h, 4ºC) and freeze

dried for EPS quantification. The dialysis solution contained 6 ppm sodium azide to avoid biological degradation of the samples. Afterwards, the EPS composition in terms of its sugar and acyl group's composition was performed. Briefly, EPS dried samples (~3mg) were dissolved in deionized water (5mL) and hydrolyzed with trifluoroacetic acid (TFA) (0.1mL TFA 99%) at 120ºC for 2h. The hydrolyzate was used for the identification and quantification of the constituent sugar and acyl group residues present in the biopolymer by HPLC.

Äkta Purifier system from GE Healthcare (Uppsala, Sweden) was used for measurement of IgG concentration in the top and bottom phases to carry out analysis. Quantification was done by using porous protein A affinity column from Applied Biosystems (Foster City, CA, USA). IgG concentration was determined from a calibration curve obtained using Gammanorm IgG as a standard.

Bradford method was used to determine total protein concentration using a Coomassie reagent from Pierce (Rockford, IL, USA). The standard for protein calibration was made with bovine gamma globulin. Absorbance was measured at 595 nm in a Spectra Max 340PC microplate reader from Molecular Devices (Sunnyvale, CA, USA).

Zeta potential measurements were performed with a Zetasizer Nano ZS system from Malvern (Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with different pH solutions of 10 mM KNO_3, ranging from pH 3 to 12. The zeta potential was calculated using the Smoluchowski equation. Hydrodynamic diameter measurements were performed with a Zetasizer Nano ZS system from Malvern (Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with milli-Q water and then used for measurements.

4. 3 Results and discussion

4.3.1. Enterobacter A47 and EPS production

Enterobacter A47 adapted the bioreactor Medium E* supplemented with glycerol as carbon source in a time period of around 6h and entered an exponential growth phase that ended in ~ 20h.The time period at which the ammonium concentration became limiting (under 0.1 g NH_4^+ L^{-1}), the fed-batch phase was initiated with the addition of mineral medium with high glycerol concentration (200 g/L), at a constant rate (10mL/h).Ammonium concentration was thereafter kept at a residual value (below the detection limit), even though the feeding solution containing 0.9 g NH_4^+ L^{-1} was fed to the bioreactor, while the DO concentration was controlled at 10% by the automatic variation of the stirring speed between 300 and 800 rpm. Cultivation run of Enterobacter-A47 shown in figure-1 which gives details of cell growth on glycerol and ammonium as well as EPS production over time.

The culture attained a maximum cells dry weight (CDW) of 6.88 g/L, at the end of the exponential growth phase (~20h). Subsequently, a slight decrease of the CDW was observed, which could be related with a loss of cell viability due to ammonium and oxygen limiting conditions imposed in the bioreactor. Furthermore, it could be also a result of dilution of biomass caused by volume withdrawn from the bioreactor for sampling with concomitant continuous feeding of fresh medium and pH control solutions,

since bacterial cells were no longer multiplying at this stage [27]. Concomitant with cell growth, glycerol concentration in the culture broth decreased from the initial 23.787 g/L to 15.207 g/L by the time the fed-batch phase was initiated (Figure 4.1).

Regarding EPS synthesis, it was initiated at the onset of the exponential growth phase, but increased production was observed during the stationary growth phase (Figure 4.1) attaining a maximum concentration of 5.25 g/L at the end of the cultivation run (94h).

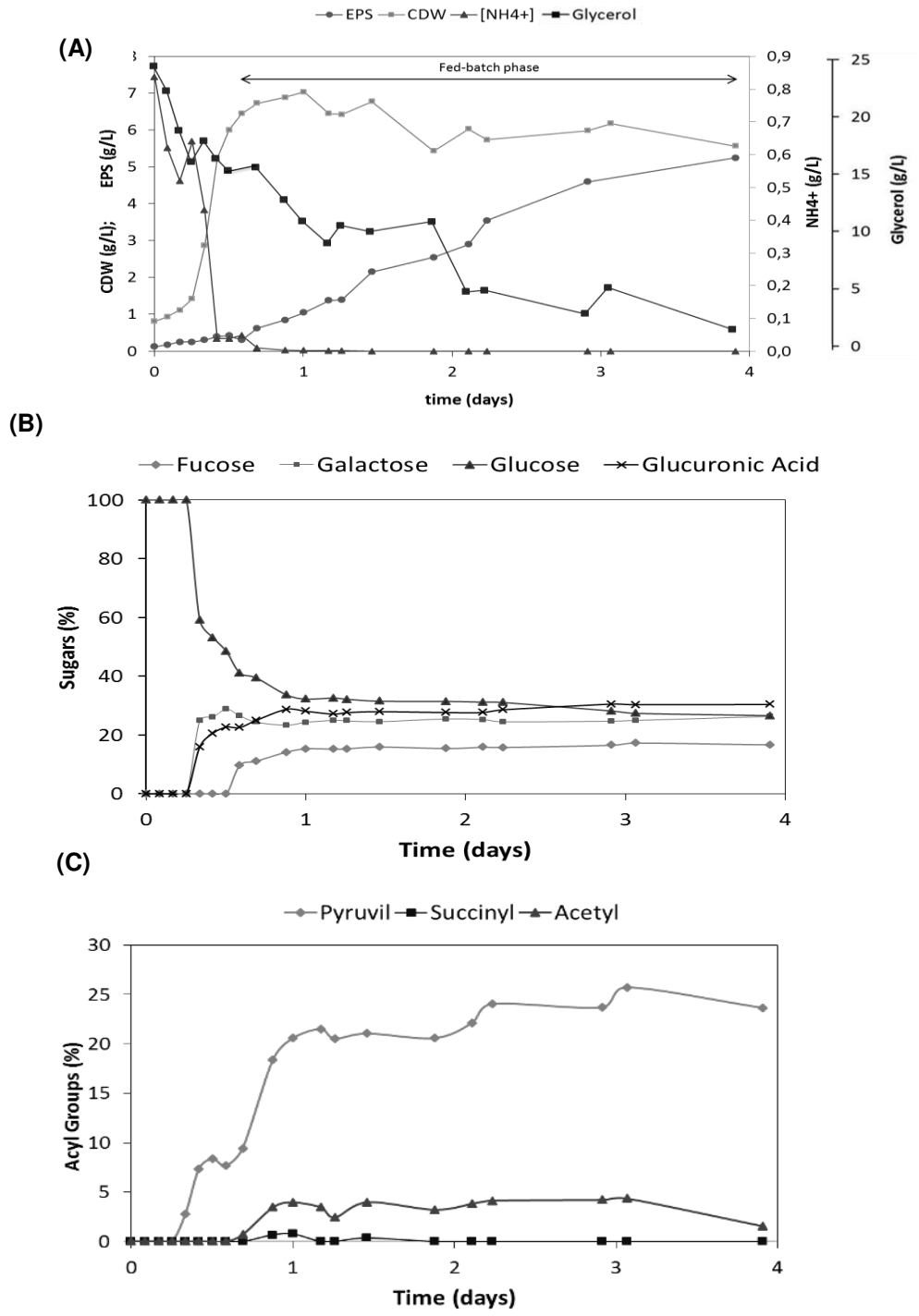


Figure 4.1. (A) Time course of the cultivation of *Enterobacter* A47 on glycerol: exopolyssacharide (EPS) concentration, Cells dry weight (CDW), ammonium concentration (NH_4^+), and glycerol concentration (glycerol). **(B)** Profile of the fucose-containing EPS sugar composition **(C)** Profile of the acyl groups during the cultivation run.

In this study, the method used was diafiltration which enables a complete ash removal and a higher protein removal than the acetone extraction method used by Alves et al. and also it is more reliable and faster than dialysis method used by Freitas et al. The viscosity of the culture broth was evaluated along time at different shear rates. At the beginning of the cultivation, the viscosity of the culture broth was 1.15 cP at 60 rpm. Whereas at the end of the run, the maximum viscosity obtained was $1.77x10^3$ cP at 0.3 rpm. The viscosity increase at the end of run may be related to increase of EPS in the broth, as well as with changes of the biopolymer's composition and molecular weight.

Figure 4.1(B) shows, the relative proportion of sugar monomers that undergone some changes throughout the cultivation run. During 24h of cultivation, glucose was the main sugar monomer of the EPS, with a content of 32.3 wt. %. Glucuronic acid, galactose and fucose were present in slightly lower amounts (28.1, 24.4 and 15.3 wt. %, respectively). After the first day of cultivation, the sugar monomers composition of EPS do not have significant changes, which was composed of fucose (16.7 wt. %), galactose (26.3 wt. %), glucose (26.6 wt. %) and glucuronic acid (30.4 wt. %). The sugar monomer composition of the EPS produced in this project was slightly different than other EPSs produced as per literature [23, 24, 25]. This behavior may reflect bacterial metabolism changes occurring throughout the run. Glucose is converted to glucose-6-P, which is one of the precursors of galactose and fucose. Therefore it would be likely to expect that the observed decreased in glucose content in the EPS composition is due to its conversion into galactose and fucose [22].Glucuronic acid, the major sugar component of our EPS, is a constituent of many different polysaccharides in plants, bacteria and animals, and is also thought to serve commonly as a protective agent, usually in the form of simple glycoside [26].

The EPS is also composed of non-saccharide components, specifically acyl groups (Figure 1C). Throughout the cultivation run the total content in acyl groups increased from 2.8 wt. % at 8h to 25.23 wt. % at the end of the run. The identified acyl groups, in the acyl hidrolysate were acetyl and pyruvil. Although succinyl was present in very low amounts at 24h, its content was 0 wt. % at the end of cultivation run. Pyruvil had the most significant increase throughout the run reaching the final content of 23.7 wt. %. Acetyl content had slightly changes throughout the run reaching its maximum content of 4.1 wt. % at day 3 and ending with 1.6 wt. % at day 4. In comparison with other similar studies [22, 23] there were some changes in the acyl groups composition of the EPS produced. In previous works it was studied that such changes in the substituent's content and composition have great impact on the polymer's properties, such as solubility and rheology. In particular, the EPS anionic character is

influenced by its content in pyruvil and succinyl, and the content in pyruvil it seems to be related with the culture broth viscosity [22].

4.3.2. Adsorption of EPS onto magnetic particles

The amount of EPS able to coat the surface of magnetic particles was studied. The results of adsorption isotherm on Bare-MPs were shown in Figure- 4.2 (A) and on TEOS-MPs were shown Figure-4.2 (B). The maximum quantity of EPS coating on bare MPs and TEOS coated MPs was 0.29 mg/g of particles and 0.431 mg/g respectively. The major reason behind reduction in nonspecific binding is the creation of a more hydrophilic layer around the iron oxide particles which is responsible to make the magnetic support more inert [26]. After successful coating of MPs using polymers it is also important to keep in mind that actual coating on surface is not uniform and homogeneous which expose the reactive iron oxide particle for interactions with other particles while use and cause the nonspecific adsorption of support. The stability and inertness results of EPS coated MPs provide best opportunity to utilize these particles for aqueous two phase extraction process with further functionalization using artificial ligand or target specific organic compounds like boronic acid.

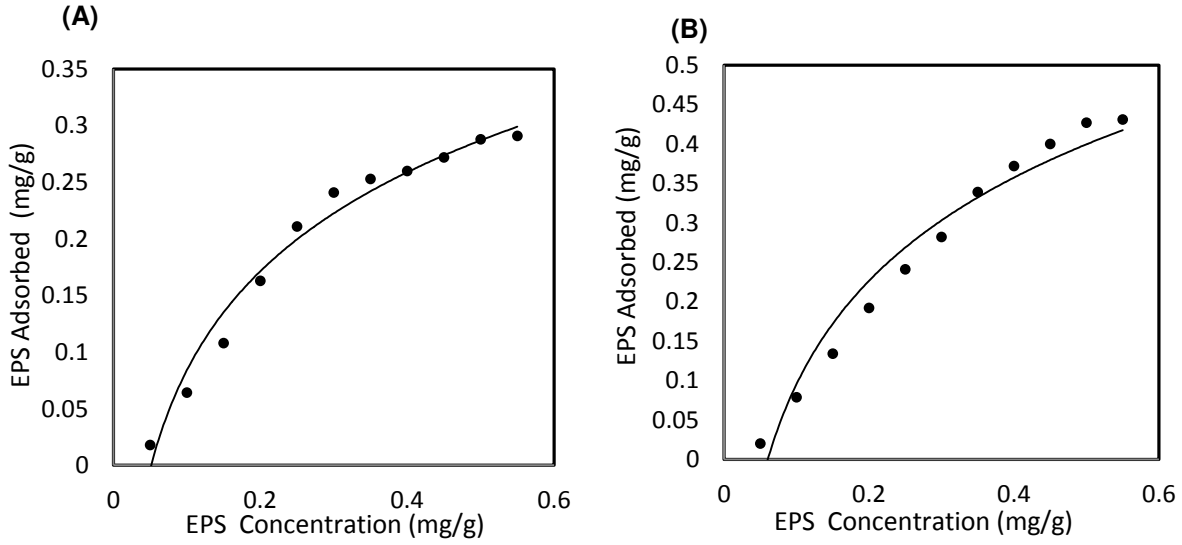


Figure 4.2. Adsorption isotherm of EPS polymer in gm of polymer adsorbed per gm of (A) Bare- MPs (B) TEOS-MPs.

4.3.3 EPS coated MPs performance in antibody purification

The produced MPs were first tested for binding to pure solutions of human IgG (hIgG) and bovine serum albumin (BSA). The MPs samples binding capacities at different stages of modification i.e. MP-Bare, MP-TEOS, MP-EPS and MP-EPS-22/8 were shown in figure-4.3(A).

Bare iron oxide (MP-Bare) particles bound 40 mg BSA,35 mg IgG/g of MP and silica coated (MP-TEOS) magnetic particles bound 82 mg BSA, 50 mg IgG/g of MP. The nonspecific binding of these MPs reduced to considerable level after coating using EPS which was in the range of 10 mg BSA and 15 mg

IgG/g of MP, this reduction in nonspecific binding is one of the important reasons behind choice of EPS polymer for coating of MPs. The MPs coated with 22/8 shows IgG binding up to 120 mg/g of MP whereas BSA binding was in the range of 8-10 mg/g of MP. The final MP-EPS-22/8 particles were then studied for static binding capacity using partition equilibrium experiments with pure solutions of hIgG.

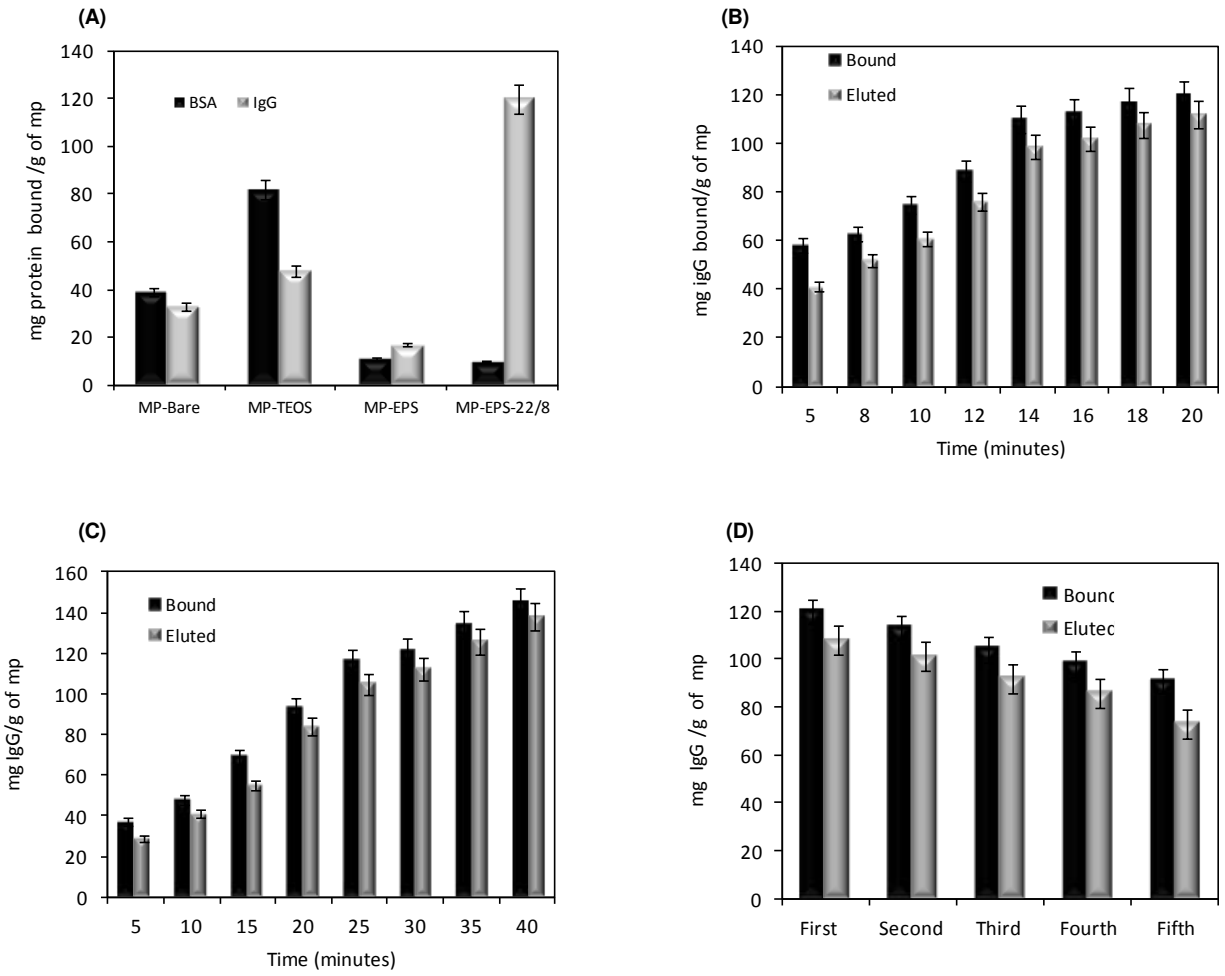


Figure 4.3. (A) Quantity of BSA and IgG bound to the support under different modification stages. MP-Bare (Fe_3O_4 magnetic particles), MP-TEOS (Tetraethoxy silane coated magnetic particles); MP-EPS (Extracellular polysaccharide coated particles), MP-EPS-22/8 (EPS coated MP modified with artificial ligand 22/8). **(B)** Regeneration results for MP-EPS-22/8 modified MP in mg of protein bound per gm of mp. **(C)** Quantity of IgG bound to the mp support at various time periods in hybrid process (ATPS plus direct magnetic fishing). **(D)** Quantity of IgG bound to the mp support in direct magnetic fishing process at various time intervals.

The adsorption isotherm of hIgG onto MP-EPS-22/8 was fitted using Scatchard plot (figure 4.4 A & B) yielding an affinity constant (Ka) of 4.3 × 10^4 M^{-1} and theoretical maximum capacity (Q_{max}) was 410 mg hIgG adsorbed/g MP. For the control MP-EPS the determined theoretical maximum capacity was 2.6 mg hIgG adsorbed/g MP (figure 4.4 C&D).

After preliminary studies with pure solutions of hIgG and BSA, the MP-EPS-22/8 supports were tested with a CHO cell culture supernatant by direct magnetic separation method, in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures. The elution samples were analysed BCA as well as Protein-A HPLC to quantify amount of antibodies. From the results it was observed that in direct magnetic separation process using complex crude mixture also magnetic supports retain their antibody binding capability and bound 122 mg IgG per g of support.

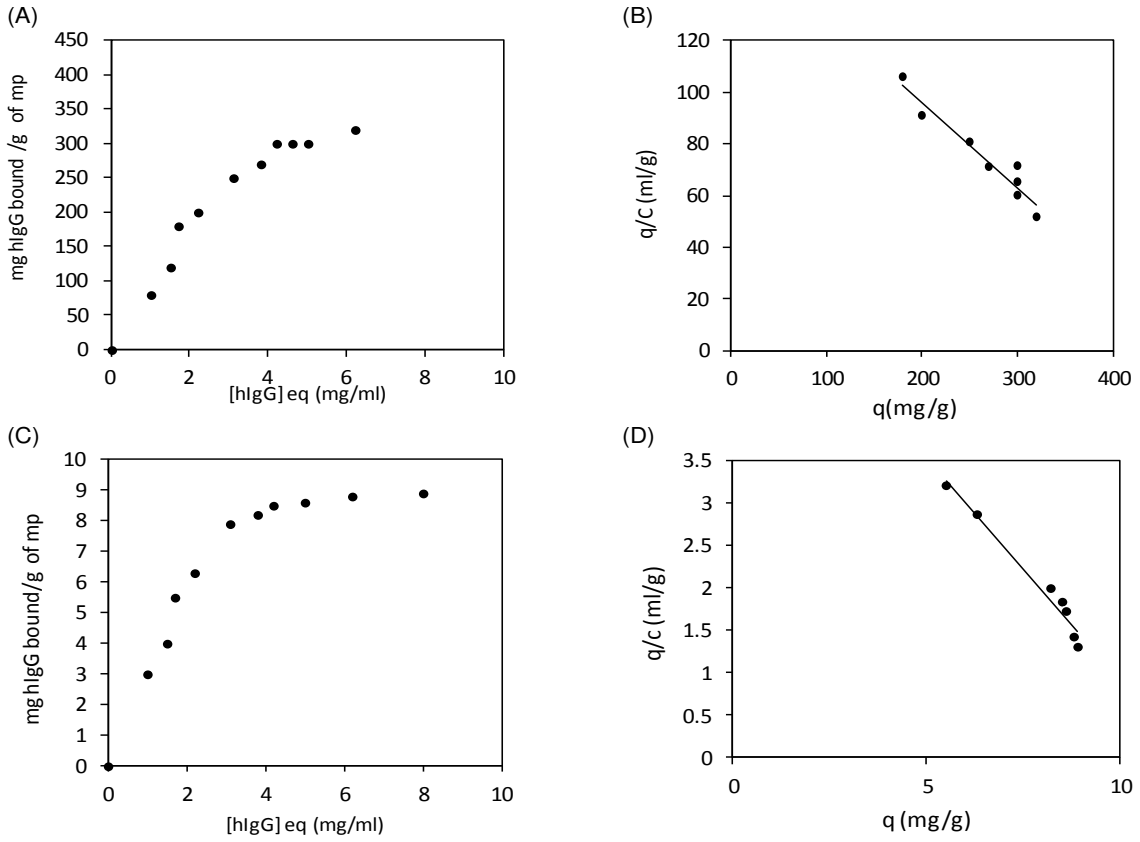


Figure. 4.4. Binding of human IgG at the surface of MP-EPS-22/8 (A; B) and MP-EPS Control (C; D). Representation of q (the amount of bound hIgG in equilibrium per mass of solid support) as function of C_{eq} (the concentration of hIgG in equilibrium). Experimental data was fitted with the expression q = (Q_{max} × C_{eq}) / (K_d + C_{eq}) using Scatchad plot, where Q_{max} corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent), and K_d is the dissociation constant. Maximum concentration of the matrix sites available to the partitioning solute.

The partition of pure IgG in PEG/Dextran ATPE supplemented with EPS coated MPs was also evaluated. As per the results shown in figure 4.5 (A), addition of salt has effect in IgG partitioning along with magnetic particles. Higher IgG concentration in upper phase was observed in presence of high concentrations of NaCl concentration. The salt concentration has been studied for 100, 200, 300, 400 and 500 mM of NaCl. It was observed that by increasing salt concentration there is decrease in the concentration of IgG in the lower dextran phase and a simultaneous increase in the concentration of IgG in the upper PEG-rich phase. After ATPE extraction step the particles were washed with washing buffer 50 mM phosphate of pH 8 whereas elution buffer was 50 mM Glycine – NaOH of pH 11. During the assessment of binding pure human IgG the bound and eluted antibody was monitored by BCA method.

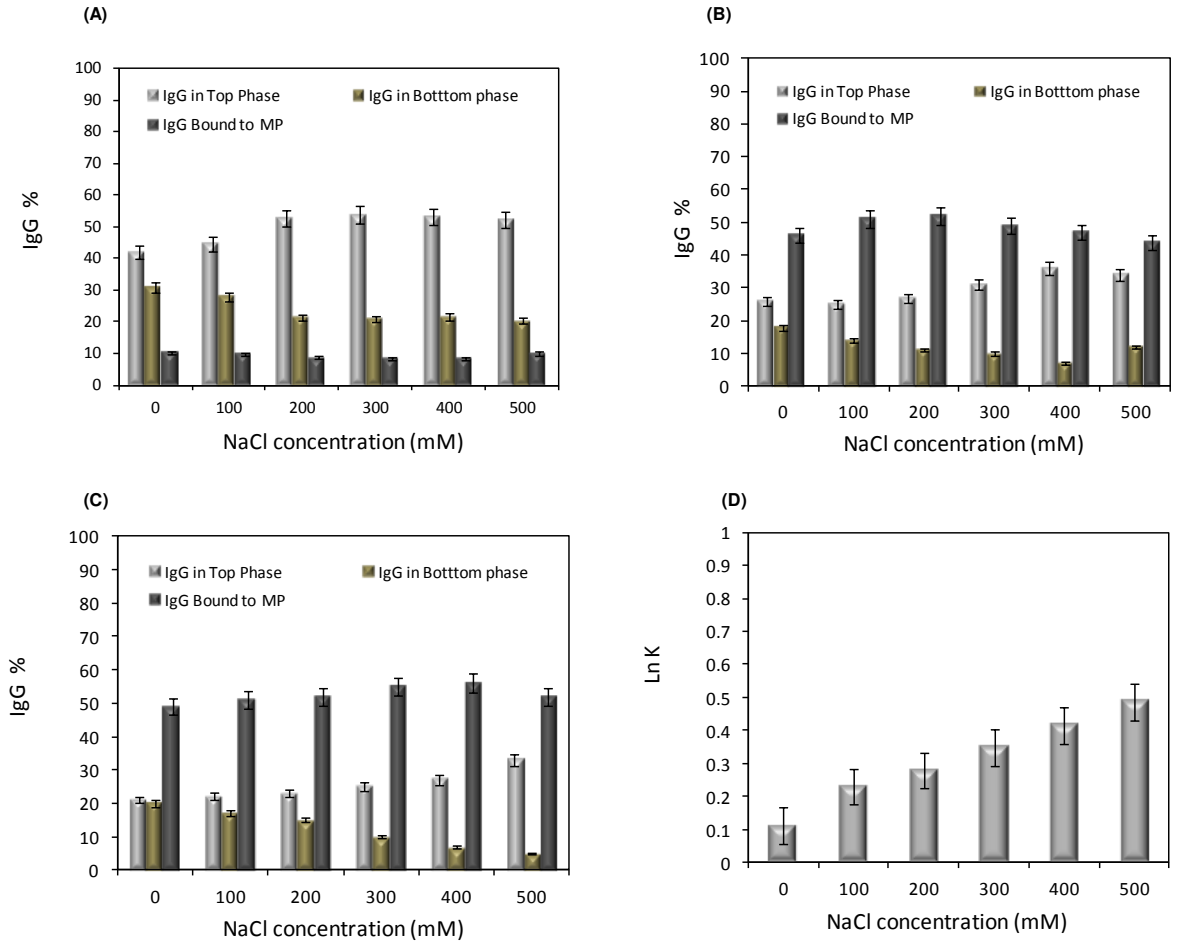


Figure 4.5. First cycle results of pure hIgG extraction in PEG/dextran systems with increasing salt concentration for (A) EPS coated MPs, (B) EPS-22/8 coated MPs, (C) First cycle Crude IgG extraction parameters and partition coefficients (D) for EPS-22/8 coated MPs.

The results are shown in figure 4.5 (A & B) for EPS-MP (Control) and EPS-22/8 MP respectively. Crude IgG partitioning and IgG binding results for first cycle of extraction using EPS-22/8 MPs were

shown in figure 4.5 (C) along with partition coefficient results (figure 4.5 D). In case of crude IgG purification sequential rise in partition coefficient was observed from 0.11 to 0.48 with increase of salt concentration from 100 mM to 500 mM.

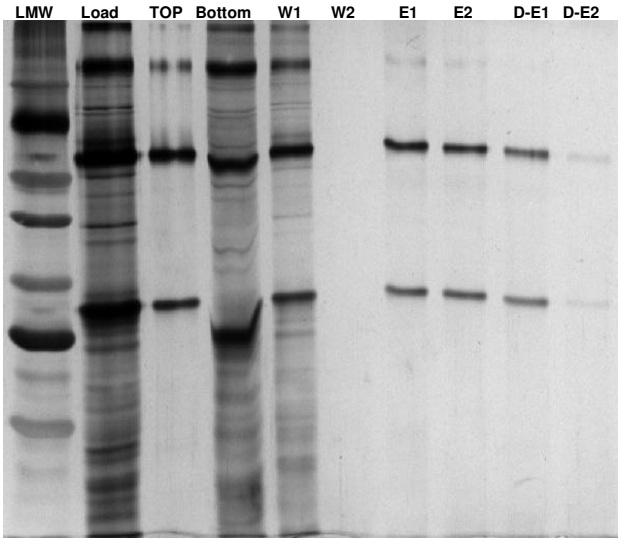


Figure 4.6. Electrophoreses gel in denaturation conditions to verify the binding capacity as well as the best elution conditions for IgG from the EPS coated MPs modified with ligand 22/8 (MP-EPS-22/8) by direct magnetic separation and ATPE method. LMW (Low Molecular Weight); Load (Loading Sample of the crude extract incubated with the adsorbent); TOP (Upper phase of ATPE system); Bottom (Bottom phase of ATPE system); W1 (First Wash with Binding Buffer-50mM Phosphate buffer of pH-8); E1 (First Elution-50mM Glycine-NaOH buffer of pH-11); E2 (Second Elution); D-W1 (First wash with Binding Buffer-50mM Phosphate buffer by direct method); D-E1 (First wash with Elution Buffer by direct method); D-E2 (Second wash with Elution buffer by direct method).

The purity of both upper and lower phases as well as elution's from magnetic nanoparticles were analysed by SDS–PAGE (figure 4.6). After confirmation by SDS-PAGE electrophoresis of the binding and elution capacity of the support on the crude extract used, the samples collected were quantified by Protein-A HPLC in order to determine the amount of protein bound to and eluted from the support.

4.4. MP-EPS-22/8 Characterization for Size, Morphology, Magnetization and Zeta.

Particle size plays important role in phase segregation as well as for good performance of magnetic particle in aqueous two phase system. The modifications using EPS polymer increases the hydrodynamic diameter of the particle. As shown in figure-4.7 (A), the average hydrodynamic diameter for EPS Coated MPs is around 1100 nm. The average hydrodynamic diameter for bare MPs varies in the range of 260-265 nm. In case of particles coated with tetraethoxysilane rise in average hydrodynamic diameter observed to 365-375 nm. The MPs with 22/8 have average diameter in the range of 970-995nm

even though some particles of size upto 3000 nm are also observed in the suspension.The morphology observation of particle using transmission electron microscopy shows formation of larger agglomerates when the particles were coated with polymers. It is observed that diameter of each individual magnetic core is in the range of 10-20 nm (figure-4.7, D&F). This might be explained by the non-covalent interactions between the coating biopolymers of neighbour nanoparticles. According to figure-4.7 C and E it is possible to conclude that the supports synthesized are not uniform in size and present a high polydispersitivity.

Through result data as shown in figure-4.7 B it was observed that zeta potential values of particle varies between -2 to -20 with variation of pH values between 3 to 12. EPS coated MPs have a zeta potential in the range of -2.5 to -17 which shows higher variation above pH 10. For EPS-22/8-MP at lower pH of 3 zeta potential value was -7.3 mV which increases gradually until pH 12 at which zeta potential value observed was -20.1.Usually, the values of zeta potential are attributed to the oxygen groups available in the tetrahedral and octahedral structure. When particles are coated with the different polymers the global charge is altered according to the type of biopolymer used. The magnetization values of both EPS-MP & EPS-22/8-MP varies between -60 e.m.u. per gm to + 60 e.m.u. per g following superparamagnetic behaviour (figure-4.7 G).

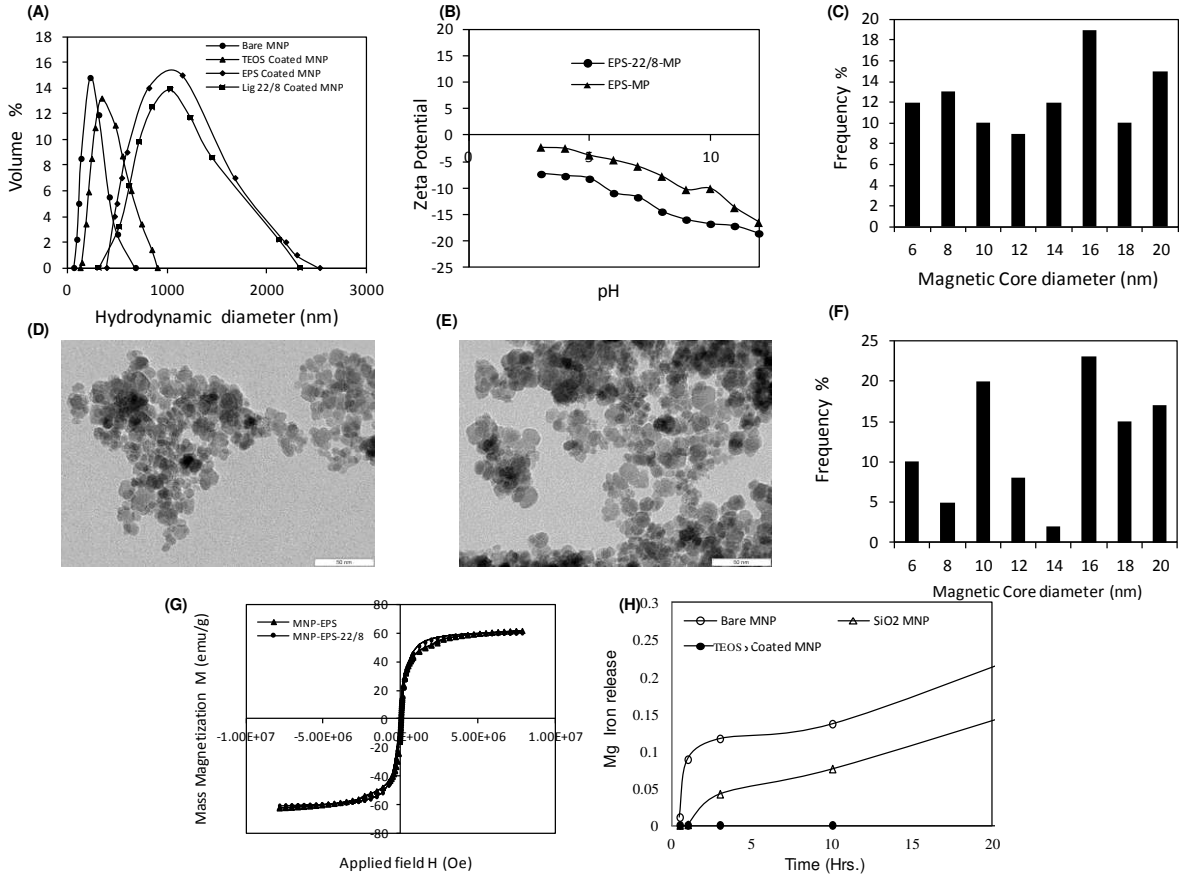


Figure 4.7. Magnetic particles characterization by DLS for Particle size distribution of MP-Bare, MP-TEOS, MP-EPS and MP-EPS-22/8 (A); Zeta potential change of EPS and 22/8 coated mp (MP-EPS-22/8) with change of pH (B); TEM image of MP-EPS magnetic particles (C); grain size distribution from TEM for MP-EPS magnetic particles (D);TEM image of MP-EPS-22/8 particles (E); grain size distribution from TEM for MP-EPS-22/8 (F); VSM curves for MP-EPS and MP-EPS-22/8 (G); Hydrodynamic diameter (F) and Zeta Potential(G).

5. Conclusion

Magnetic supports having basic core of iron oxide shows superior suitability for utilization of a fucose-containing extracellular polysaccharide produced by *Enterobacter* A47 (DSM 23139), as a coating material for MPs. EPS support found to be promising polymer for magnetic particle coating and modification due to the simplified synthesis, low cost, high stability and reduced non-specific adsorption. The excellent capability of EPS coated MPs for the covalent attachment of a synthetic affinity ligand make these particles challenging for recovery of antibodies. EPS coated MPs also shows applicability for use in integrated process technology which joins magnetic separation process with aqueous two phase extraction for the purification of human antibodies. The ATPE process composed of 8 % PEG and 5 % Dextran afforded high recovery yield in presence of EPS-22/8 coated MPs. The magnetic supports can be effectively used for five times with partial reduction in binding capacity.

References:

[1] Bruce, I. J. and Sen, T. (2005). Surface Modification of Magnetic Nanoparticles with alkoxysilanes and their Application in Magnetic Bioseparations.Langmuir 21: 7029-7035.

[2] Cakmak, S., Guemuesderelioglu, M. and Denizli, A. (2009). Biofunctionalization of magnetic poly (glycidyl methacrylate) micro spheres with protein A : Characterization and cellular interactions.Reactive & Functional Polymers 69(8): 586-593.

[3] Dhadge V.L., Hussain A., Azevedo A., Aires-Barros M. R., Roque A.C.A., Boronic acid modified magnetic materials for antibody purification, J. R. Soc. Interface 11 (2013) 20130875.

[4] Dhadge V.L., Azevedo A., Aires-Barros M. R., Roque A.C.A., Magnetic Aqueous Two Phase Fishing : An hybrid process technology for antibody purification, Submitted to Journal of Chromatography A.

[5] Ditsch, A., Laibinis, P. E., Wang, D. I. C. and Hatton, T. A. (2005). Controlled Clustering and Enhanced Stability of Polymer-Coated Magnetic Nanoparticles. Langmuir 21(13): 6006-6018.

[6] Borlido L., Azevedo AM, Sousa AG, Oliveira PH, Roque ACA, Aires - Barros MR.,Fishing human monoclonal antibodies from a CHO cell supernatant with boronic acid magnetic particles. Journal of Chromatography B, 2012. 903: p. 163-170.

[7] Grillo,M. E.,Finnis, M.W. Ranke, W. (2008). Surface structure and water adsorption on Fe_3O_4: Spin-density functional theory and on-site Coulomb interactions. Physical ReviewB77:075407.

[8] Haigh, J. M., Hussain, A., Mimmack, M. L. and Lowe , C. R. (2009). Affinity ligands for immunoglobulins based on the multicomponent Ugi reaction. J of Chromatography B877:1440–1452.

[9] Hung, C.-W., Holoman, T. R. P., Kofinas, P. and Bentley, W. E. (2008). Towards oriented assembly of poteins onto magnetic nanoparticles. Biochem. Engineering Journal 38: 164–170.

[10] JianHan, X., Hui, S., Wei, Z., Yi, L., JiaXin, D. and Günter, B. (2007). Surface chemistry of nanoscale Fe3O4 dispersed in magnetic fluids. Science in China Series B: Chemistry 50(6): 754-758.

[11] Konwarh, R., Karak, N., Rai, S. K. and Mukherjee, A. K. (2009). Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase. Nanotechnology 20(22).

[12] Kumar, M. K., Reddy, A. L. M. and Ramaprabhu, S. (2008). Exfoliated single-walled carbon nanotube-based hydrogen sensor.Sensors and Actuators B 130: 653–660.

[13] Lattuada,M.and Hatton,T. A.(2007).Functionalization of Monodisperse Magnetic nanoparticles. Langmuir 23: 2158-2168.

[14] Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Elst, L. V. and Muller, R. N. (2008). Magnetic Iron Oxide Nanoparticle : Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications. Chemical Reviews 108: 2064–2110.

[15] Li, J. and Gao, H. (2008). A Renewable Potentiometric Immunosensor Based on Fe3O4 Nanoparticles Immobilized Anti-IgG. Electroanalysis 20(8): 881-887.

[16] Liang,Y.Y., Zhang, L.-M., Li, W. and Chen, R.-F. (2007). Polysaccharide-modified iron oxide nanoparticles as an effective magnetic affinity adsorbent for bovine serum albumin. Colloid Polym Sci 285: 1193–1199.

[17] Lu, A., Salabas, E. L. and Schüth, F. (2008). Magnetic Nanoparticles: Synthesis, Protection, Functionalization, and Application. Angewandte Chemie International Edition 46: 1222 – 1244.

[18] Park, C., Park, K. H., Kwon, D. and Yoon, T. H. (2008). Biocompatible Quantum Dot Nanocolloids Stabilized by Gum Arabic. Bulletin of the Korean Chemical Society 29(6): 1277-1279.

[19] Roque,A.CA, Bicho A, Batalha IL, Cardoso AS, Hussain A. (2009). Biocompatible and bioactive gum arabic coated iron oxide magnetic nanoparticles. Journal of biotechnology. 144 (4): 313-320.

[20] Santana D. F., Dhadge V. L., Roque , A.C.A ., Dextran coated magnetic supports for antibody purification. ACS Appl. Mater. Interfaces, 2012. 4(11): p. 5907-5914.

[21] Cruz, M., Freitas, F., Torres, C.A.V., Reis, M.A.M., and Alves, V.D. Influence of temperature on the rheological behavior of a new fucose-containing bacterial exopolysaccharide. International Journal of Biological Macromolecules.48, 695-699, 2011.

[22] Torres, C.A.V., Marques, R., Antunes, S., Alves, V.D., Sousa, I., Ramos, A.M., Oliveira, R., Freitas, F., and Reis, M.A.M. Kinetics of production and characterization of the fucose-containing exopolysaccharide from enterobactor A47. Journal of biotechnology. 156, 261-267, 2011.

[23] Freitas, F., Alves, V.D., Pais, J., Costa, N., Oliveira, C., Mafra, L., Hilliou, L., Oliveira, R., and Reis, M.A.M. Characterization of an extracellular polysaccharide produced by a Pseudomonas strain grown on glycerol. Bioresource technology.100, 859-865, 2009.

[24] Freitas, F., Alves, V.D., and Reis, M.A.M. Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends in biotechnology.29:8, 388-397, 2011a.

[25] Freitas, F., Alves, V.D., Torres, C.A.V., Cruz, M., Sousa, I., Melo, M.J., Ramos, A.M., and Reis, M.A.M. Fucose-containing exopolysaccharide produced by the newly isolated Enterobacter strain A47 DSM 23139. Carbohydrate polymers.83, 159-165, 2011b.

[26] Cimini, D., De Rosa, M., and Schiraldi, C. Production of glucuronic acid-based polysaccharides by microbial fermentation for biomedical applications. Biotechnology Journal, 7:2, 237-250, 2012.

[27] Shamim, N., et al., , Adsorption, desorption, and conformational changes of lysozyme fromthermosensitive nanomagnetic particles. Journal of Colloid and Interface Science, 2008. 320(1): p.15

CHAPTER 5

LIGAND A2C7I1 MODIFIED MAGNETIC PARTICLES FOR BIOPURIFICATION

SUMMARY

Antibodies are pharmaceutical market thrust area having exponential growth during previous decade. Growing medicinal applications drives worldwide researcher's attention for developing and purifying effective antibody fractions for human beings. As major bottleneck of antibody manufacturing industry relies on effective purification methodologies process development is one of the major challenge from future perspective. Nowadays magnetic nanoparticle based process technologies found to be one of the suitable option from process development point of view. High gradient magnetic separation based technology provide selective recovery as well as high yield method for purifying monoclonal antibodies from cell cultures. In this work magnetic nanoparticles synthesized by co-precipitation method were coated with an affinity ligand for the selective separation and the purification of monoclonal antibodies. Magnetic materials synthesized using coprecipitation method were initially modified with oxidized dextran and then functionalized with an Ugi based ligand for selective antibody purification. The main role of coating particles with dextran is to reduce non-specific binding and providing aldehyde groups (due to oxidation of sugar moieties) on MPs surfaces for ligand modification. Synthesized particles were able to adsorb 1.5 mg dextran/g of MP at low concentration, whereas 2.2 mg dextran/g of MP adsorption observed at high concentration. The ligand modified particles were tested with crude antibody extracts which able to bind 120 mg of IgG per gram of magnetic support and able to elute 90 mg under mild conditions.

5.1. Material and Methods

5.1.1 Materials

1-amino-2-propanol(93%), 2-(Ethylthio)ethylamine, (3-Aminopropyl)triethoxysilane 99%, 3-(Ethylthio)propanoic acid , Albumin bovine serum min 98% electroph. Alpha-D-Glucose, anhydrous, 96%, Anthrone, APTES (3-Aminopropyl) triethoxysilane, Bicinchoninic acid, Citric Acid, Copper(II) Sulfate, Dextran from *Leuconostoc mesenteroides* , Human immunoglobulin IgG from Octapharma, Phenol, Ferric sulphate heptahydrate, Ferric sulphate hexahydrate, Glutaraldehyde solution 50 wt% in H_2O, Gum Arabic from Acacia tree, Iron(III)chloride hexahydrate, Potassium cyanide ≥ 96%, Potassium hydroxide, Pyridine > 99% , Sodium carbonate, Sodium metasilicate pentahydrate, Sodium hydrogencarbonate, Sodium periodate, Sodium phosphate monobasic monohydrate, Succinamic acid, 97%, Sulfuric Acid, Tetraethyl orthosilicate, were supplied by Sigma-Aldrich. Ascorbic Acid was supplied by Fisher Scientific .Ehanol 96%, Ethanol Absolute PA, Ethylene Glycol, Sodium acetate, Sodium di-hydrogen phosphate 1-hydrate, Sodium Hydroxide were supplied by Panreac. Glycine was purchased from Acros. Ammonium hydroxide, Ninhydrin and TTC (2,3,5-Triphenyl-tetrazolium chloride solution) were purchased from Fluka. Hydrochloric Acid, Methanol>99% were supplied by CarlRoth.

Materials used were Dialysis sleeves MWCO 10000 Da, Microtest plate 96-well Transparent (Sarstedt), deep well plates PP rectangular 2,5 ml. Laboratory equipament were magnetic support, Selecta orbital Shaker, Microplate Reader Infinite F200 Tecan, pressure containes, water bath SHC 2000, Scanvac, mini-micro centrifuge Scan Speed, Bandelin Sonorex super RK25577, Lenton laboratory chamber furnace(1200°C).

5.1.2 Methods

5.1.2.1 Oxidation of dextran in solution (Dx-Ox) followed by magnetic particles coating

The oxidation of dextran was performed using 1.25 g sodium periodate for a 50 ml solution of dextran (40mg/ml). The solution was stirred magnetically at room temperature in the dark for 6 hours. After the reaction, contents were dialysed (MWCO 1000) against water for 48 hours with several changes of water unitl the dialysate was free from periodate.

Dextran has proven to be one of the most commonly utilized coating agents, because of neutral nature and easy to coat property. In addition, dextran has shown to be ideal to generate magnetic particles for bioseparation. For oxidized dextran coating, the MPs (5mg/ml) were recovered from water and resuspended in the oxidized dextran solution. The mixture was stirred for 2 hours at room temperature and washed 5 times with distilled water.

5.1.2.2 Functionalization of magnetic nanoparticles with Glutaraldehyde

Glutaraldehyde has been used extensively as a functional crosslinking reagent. The glutaraldehyde reacts by several routes to form covalent crosslinks with amine-containing molecules. As such, it is possible to functionalize the aminated MPs.

The particles (5 mg/ml) were washed with distilled water and then dispersed in 50 ml of PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) containing 20 ml 25% glutaraldehyde, and left under mechanical stirring for 30 min. Finally, the resultant MPs were washed several times with distilled water.

5.1.2.3 Ligand A2C7I1 Modification

Based on Ugi Reaction, two types of MPs were tested, one functionalized with glutaraldehyde and other functionalized with oxidized dextran. The four-component reaction of synthesis process consists of reaction of MPs functionalized with aldehyde in methanol with an equimolar amount of 4-aminobenzamide in methanol, and the solution was left to stirr for 1 hour at room temperature. An equimolar amount of the carboxylic acid C7 (4-hydroxyphenylacetic acid) in methanol and isopropyl isocyanide were then added to the solution and left to stir for 48 hours at room temperature.

5.1.2.4 Ligand with sulfur modification by Ugi Reaction

The Ugi reaction was first reported by Ivar Ugi in 1959 and it is a multi-component reaction in organic chemistry involving a ketone or aldehyde, an amine, an isocyanide and a carboxylic acid. The Ugi reaction has often been used as a tool in the synthesis of pharmacologically active molecules, natural products, therapeutic agents, and combinatorial libraries.

The magnetic nanoparticles functionalized with oxidized dextran act as aldehyde molecule, due to the presence of aldehyde on the nanoparticle surface. For the Ugi reaction, the quantities of compounds were calculated on the basis of estimated aldehyde content using Sabolks method. The reagents used were 5 times excess the value obtained by analysis.

To determine the concentration of ligands on MPs surface, the compounds that were used have sulfur in their composition. By Inductively Coupled Plasma Mass Spectrometry (ICP-MS), it is possible to determine the amount of sulfur in the solution, it follows that it is able to calculate the quantities of ligand.

5.1.2.5. Direct magnetic fishing of antibodies

The functionalized MP-A2C7I1 were tested with a pure solution of hIgG, and with a pure solution of BSA. The particles suspensions were washed firstly with 0.1 M NaOH prepared in 30% (v/v) isopropanol, followed by washing with deionized water. After washing the nanoparticles suspensions were equilibrated with 50 mM phosphate buffer of pH 8. After washing and equilibration of support the supernatants were removed and added with 500 µl of a hIgG or BSA solution of 1 mg/ml concentration diluted in 50 mM phosphate buffer. The incubation was carried out for 15 minutes at room temperature with constant stirring. The supernatant was then collected and the particles were washed five times with

500 µL binding buffer. After preliminary washing steps elution of bound protein was then carried out using various buffer solutions of different pH range. All samples were analyzed by BCA assay with microplate reader in order to quantify the amount of protein bound to and eluted from the supports. In order to compare results the non-modified particles and ligand modified particles were tested at the same time and in the same conditions of the magnetic supports.

5.1.2.6. Regeneration and reuse of MP-A2C7I1

MP-A2C7I1 were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The A2C7I1 functionalized magnetic particles having concentration of 10 mg/ml were tested with 500 µl of each of the pure hIgG solution, by incubating for 15 minutes at room temperature. The liquid in which particles were suspended were removed by magnetic separation and then washed two times with 500 µl binding buffer. After washing, MPs were eluted five times using 500 µL elution buffer. All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

5.1.2.7. Studies of incubation time for direct separation process

Incubation time plays important role to adsorb antibody on the surface of magnetic particles. Functionalized MP-A2C7I1 were studied in order to finalise exact incubation time to be provided to have optimum separation ability. This study was carried out separately for direct magnetic separation. For direct magnetic separation incubation time study five different test samples of MP-A2C7I1 having 500 µl volume were incubated at room temperature with 500 µl hIgG solution (1 mg/ml) for different time intervals mainly 5,8,10,12,14,16,18 and 20 minutes respectively. Incubation was followed by washing the particles two times with 500 µl binding buffer and five times elution using elution buffer, in order to know maximum hIgG that we able to elute. All washes were collected and quantified by BCA method.

5.1.2.8 Crude IgG extracts purification

Magnetic supports MP-EPS-22/8 were tested with CHO cell culture supernatants in order to estimate efficiency of MPs for crude purification. In case of direct method, 500 µl MP-Dx and MP-A2C7I1 with a concentration of 10 mg/ml were incubated with 500 µl of the crude extract respectively, without any pre-treatment, for 20 minutes at room temperature. After incubation the particles were separated and supernatants were collected. The separated particles were then washed five times with 500 µl binding buffer, After washing, MPs were eluted using elution buffer in order to study the best elution conditions. All collected samples were quantified by affinity chromatography using Äkta purifier system from GE Healthcare (Uppsala, Sweden), using porous protein A affinity column from Applied Biosystems (Foster

City, CA, USA). The BCA method and gel electrophoresis (12.5% Acrylamide/Bisacrylamide) in denaturing conditions by SDS-PAGE was also used for further analysis. The respective gels were prepared according to a standardized protocol. The low molecular weight marker and the samples for running gels were prepared by adding 2.5 and 10 µl of each sample, respectively, as well as 5 µl of sample buffer and boiled for two minutes immediately before applying to the gel. The gel was run for 80 minutes at 150 V and 250 mA by adding an electrophoresis Tris – Glycine Buffer (SDS-PAGE). For detection of the protein bands, the gel was stained with silver stain Biorad kit.

5.2 Analytical techniques

5.2.1 Determination of Aldehydes by Sabolks Method

This method is based on the ability of aldehyde groups to reduce 2, 3,5 triphenyl-2H-tetrazolium chloride (TTC) leading to the formation of a red dye called formazan, which can be determined by spectrophotometry in the UV-visible. This method is also known as Sabolks method and the reaction is described below.

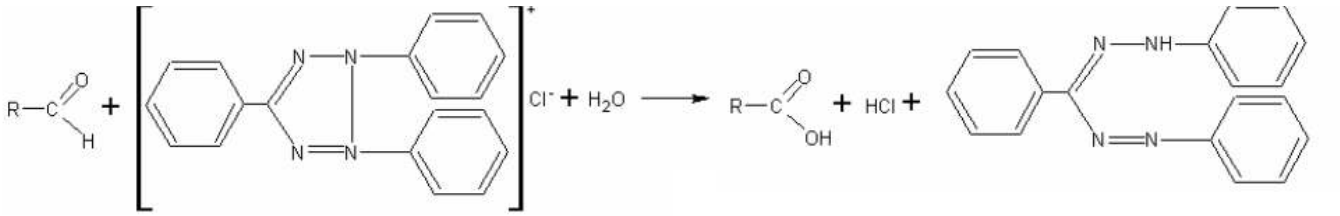


Figure 5.1. Reaction of aldehyde group with TTC (2,3,5,triphenyl-2H-tetrazolium chloride

A calibration curve was plotted using glucose solutions (10, 5, 2.5, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001 mg/ml). The procedure consists of the addition of 500 µL solution of sample, followed by 50 µL KOH 0.2M and 50 µL of TTC (0.002 g/ml). The mixture is boiled at 100°C, then allowed to cool. The solutions were transferred to a microplate (200 µL) and the absorbance is measured at 560 nm.

5.2.2 Zeta Potential and Dynamic Light Scattering (DLS)

Zeta potential measurements were performed with a Zetasizer Nano ZS system from Malvern (Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with different pH solutions of 10 mM KNO_3, ranging from pH 3 to 12. The zeta potential was calculated using the Smoluchowski equation. Hydrodynamic diameter measurements were performed with a Zetasizer Nano ZS system from Malvern

(Worcestershire, UK). Particle suspensions were diluted to 0.005 wt% with milli-Q water and then used for measurements. DLS analysis also carried out using Zetasizer Nano ZS from Malvern. Samples were prepared in milli-Q water having final particle concentration of 0.05 mg/ml. This method was used for the characterization of magnetic nanoparticles regarding hydrodynamic diameters.

5.2.3 Inductively coupled plasma mass spectrometry (ICP-MS)

Inductively coupled plasma Mass Spectrometry or ICP-MS is an analytical technique used for elemental determinations. The sample is introduced into the ICP plasma as an aerosol, either by aspirating a liquid or dissolved solid sample into a nebulizer or using a laser to directly convert solid samples into an aerosol. Once the sample aerosol is introduced into the ICP torch, it is completely desolvated and the elements in the aerosol are converted first into gaseous atoms and then ionized towards the end of the plasma.

5.3.Results and Discussion

5.3.1 Magnetic nanoparticles coating performance

Anthrone test was used to determine the amount of dextran coated at MPs surface. Standard graph prepared using several dextran dilutions was used to estimate dextran concentration using linear regression. After dextran coating the particles were washed with distilled water. Thus, the particles coated with dextran were washed and the absorbance of each wash was measured at 600nm. The difference between the total amount of dextran used initially for coating and the total amount of dextran in the washes is the total amount that is coated at MNP surface. Two concentrations of dextran were tested in three types of particles synthesized by solvothermal method. It was used 50 ml solution with a concentration of approximately 5mg of particles/ml.

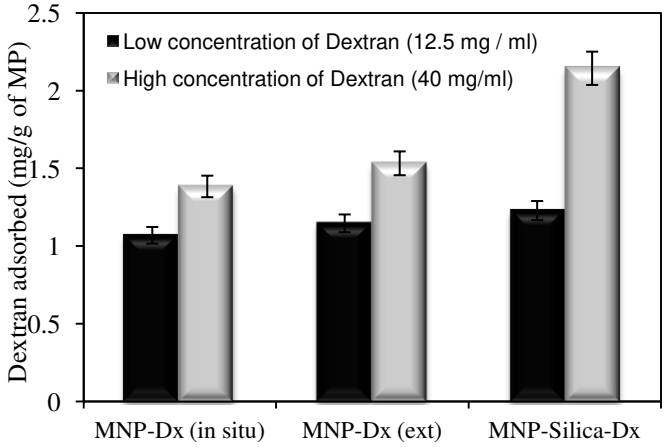


Figure 5.2. Representation mg of dextran bound/ mg MP for two types of concentrations

The quantities of dextran coated on the surface of Dx(in situ) coated MPs were 1.1 mg/g of MP for low concentration and 1.4 mg/g of MP for high concentration. For Dx(ext)-coated MPs, the amount was 1.4 mg/g of MP for low concentration and 1.5 mg/g of MP for high concentration. Finally, MP-EG-Silica-Dx showed a coating with 1.2 mg/g of MP for low concentration and 2,2 mg/g of MP for high concentration of dextran.

Particles coated with silica followed by dextran coating showed the best results, probably, due to a creation of a spongy adsorptive surface with high adsorption capacity to polymeric substances. Also, coating with dextran externally has been shown to be more effective than coating *in situ* (coating during the reaction).

5.3.2 Nonspecific binding study of magnetic supports

BSA was used as standard non-specific binding protein in order to know the quantities that MPs adsorbs on surface. This study is important in order to judge the capability of developed MPs for undesired protein adsorption from complex crude mixtures.

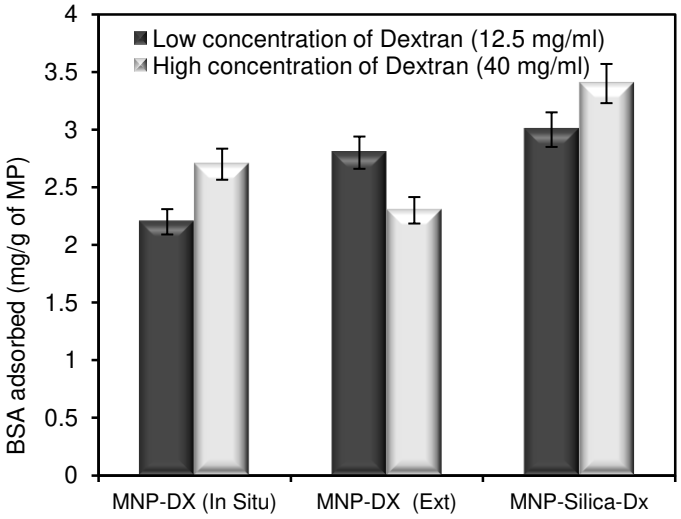


Figure 5.3. Adsorption of BSA on MPs synthesized by coprecipitation method

The particles without any coating adsorbed a total amount of 1.35 mg of BSA/g of MP. For low concentration of dextran, particles coated with dextran *in situ* and dextran after the reaction absorbed 2.14 mg of BSA /g of MP and 2.89 mg BSA/g of MP, respectively. For high concentration of dextran, MP-Dx(in situ) adsorbed 2.66 mg of BSA/g of MP and MP-Dx(ext) adsorbed 2.38 mg of BSA /g of MP. Particles coated with silica and dextran showed a adsorption of 3.075 mg/g of MP and 3,485 mg/g of MP for low and high concentration of dextran, respectively. It is observed that silica coating increases the adsorption of non-specific protein. Also, the particles coated with dextran in two different ways showed results at the same range.

5.3.3 Size distribution

The size distribuition for each type of particles was measured by DLS, which is common analytical techniques and the associated range scale involved for nanoparticle sizing. The next four figures represent the size distribution of MP-EG, MP-EG-Dx(in situ), MP-EG-Dx(ext) and MP-Silica-Dx, respectively. The size of MPs is one of the parameter that often required due to many possible applications, because each application performs better with a particle having a certain range size. The average size of particles without any type of coating was found to be 963 nm. The particles coated with dextran in situ while synthesis shows average particle size of 1530 nm. In case of particle coated with stirring shows slightly higher size than those coated in situ, average particle size in this case was in the range of 1830 nm. For silica coated particles there was sharp rise in particle size which shows average range of 1980 nm.From above results it was observed that coating increases the size of particle due to the formation of layer. Silica coating provides the larger increase and dextran-coated (in situ) MP has a higher size than MP coated with dextran after the reaction.

5.3.4. Zeta Potential

In parallel, zeta potential was measured for all suspensions and determined approximate isoelectric points in order to determine the charge on the surface of particles. DLS was used to obtain these results. The average zeta potential of Bare MNP was -45.8 mV, this zeta reduces to near neutral level after coating with dextran which is a neutral polymer. In case of in situ dextran coating zeta potential was -0.0146 while in case of externally dextran coated particles zeta potential found to be -0.0988 (mV).

5.3.5. Magnetization curve

The magnetization curve aims to analyze the magnetic properties of particles. For biopurification, it is necessary to have superparamagnetic properties. This phenomenon occurs with materials that need to be under the action of a magnetic field in order to have better magnetic response. When the MPs were separated from magnetic field, the magnetization disappears.

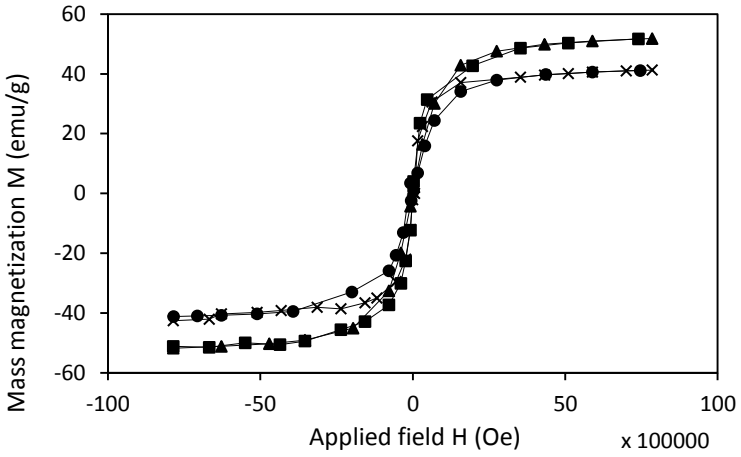


Figure 5.5 Magnetization curves for four types of particles

As it is observed from the graph, both types of particles present superparamagnetic properties. As such, the particles can be dissolved in solution without the presence of a magnet.

5.4 Adsorption isotherm of Dextran and Dextran oxidize on Bare and silica coated MPs

The particles synthesized by solvothermal as well co-precipitation method were studied for adsorption of dextran and oxidized dextran. For MP-Bare, maximum amount of dextran that adsorbed on the surface is 30 mg of dextran/g of MP for 12 hours of agitation. For 2 hours of agitation, maximum quantity that gets adsorbed was 18 mg/g of MP. When we replace polymer using oxidized dextran, the maximum amount adsorption found to be 35 mg of oxidized dextran/g of MP. The results were shown in Figure 5.6. The particles coated with silica show higher adsorption values as compared to MP-EG. As shown in Figure 30, the maximum amount of dextran adsorbed on magnetic particles was 45 mg of dextran/g of MP, but for oxidized dextran, the adsorption value less.

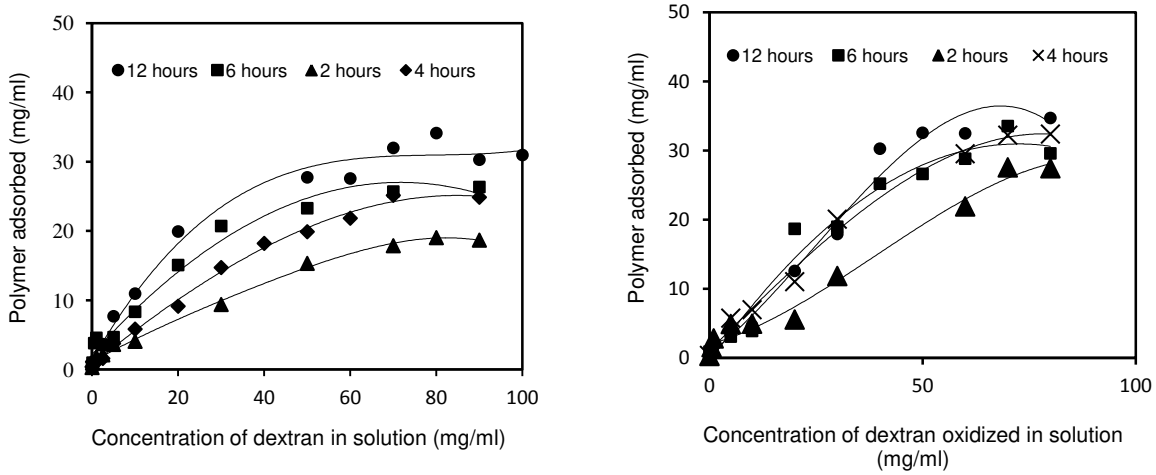


Figure 5.6. Adsorption isotherm of dextran and oxidized dextran on MP-Bare

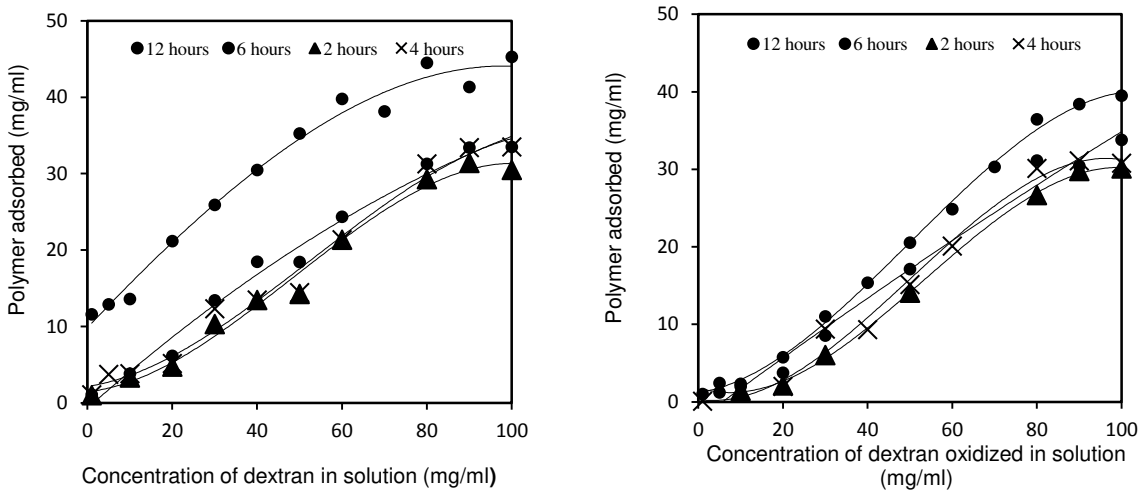


Figure 5.7. Adsorption isotherm of dextran and oxidized dextran on MP-Silica

5.5.Quantification of aldehydes

Sabolks method was applied for aldehyde content determination. This method was used to quantify the carbonyl groups of oxidized dextran before and after the periodate oxidation. For each mol of glucose, there is one mol of aldehyde. As such, 1mg of glucose/ml represents approximately 5.5 µmol of aldehydes/ml. The calibration curve expressed in µmol of aldehyde/ml vs. Absorbance is used to calculate the concentration of aldehyde in the samples.

$$C_{aldehydes}(\mu mol/ml) = \frac{A - 0.0041}{0.0239}$$

Different concentration of dextran were oxidized and tested with Sabolks method. Also, control solution was tested with dextran solutions without oxidation.

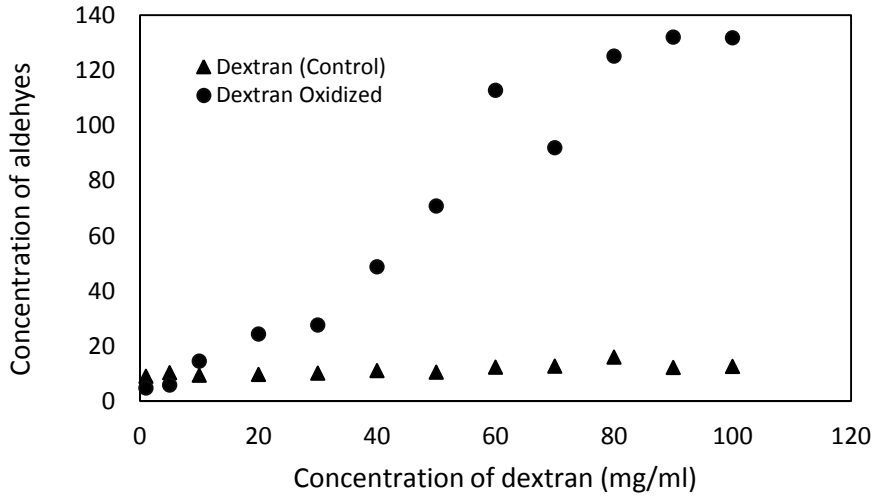


Figure 5.8. Quantification of aldehydes by Sabolks method with control solutions

Thus, it is possible to conclude that Sabolks is a valid method to quantify the amount of aldehydes in a Dextran solution. It is necessary to know the concentration of aldehydes because the Ugi Reaction is based on an equimolar solutions of 4 components. MNPs functionalized with glutaraldehyde and dextran oxidized were testes with Sabolks method. The particlesfunctionalized with glutaraldehyde showed 2.7 µmol of aldehydes/ml and particles functionalized with dextran oxidized showed 3.1 µmol of aldehydes/ml of MPs.

5.6 A2C7I1 coated MPs performance in antibody purification

The produced MPs were first tested for binding to pure solutions of human IgG (hIgG) and bovine serum albumin (BSA). The MPs samples binding capacities were shown in figure-5.9. Final modified magnetic particles bind 20 mg of BSA per gram of MP whereas able to elute around 12 mg of BSA per gm of MP which is one of the effective and beneficial property shown by these particles as we need as less BSA binding as possible for application purpose. IgG binding was upto 130 mg per gram of MP whereas elution was too less. This elution difficulty study results were shown in next topic for optimization to obtain effective buffer for better product recovery. The final MP-A2C7I1 particles were then studied for static binding capacity using partition equilibrium experiments with pure solutions of hIgG.

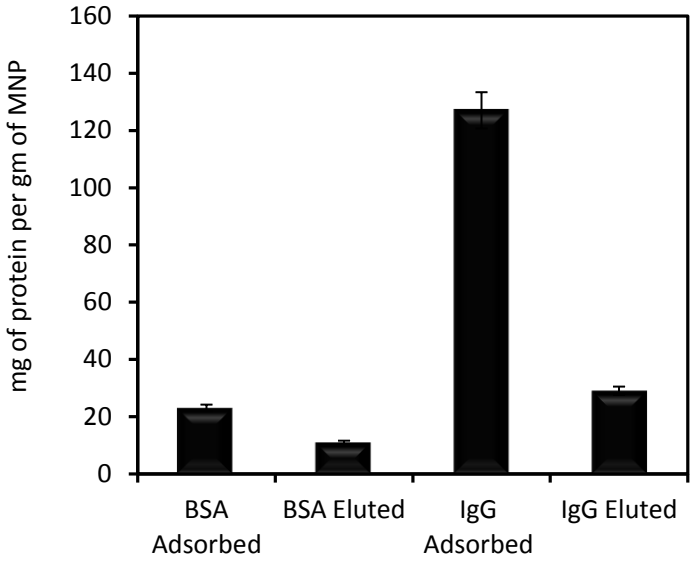


Figure 5.9 Quantity of BSA and IgG bound to the support

The adsorption isotherm of hIgG onto MP-A2C7I1 was fitted using Scatchard plot yielding an affinity constant (Ka) of $3.3 \times 10^4\ M^{-1}$ and theoretical maximum capacity (Q_{max}) was 275 mg hIgG

adsorbed/g MP. For the control MP-DX-Ox the determined theoretical maximum capacity was 2.6 mg hIgG adsorbed/g MP. After preliminary studies with pure solutions of hIgG and BSA, the MP-A2C7I1 supports were tested with a CHO cell culture supernatant by direct magnetic separation method, in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures. The elution samples were analysed BCA to quantify amount of antibodies. From the results it was observed that in direct magnetic separation process using complex crude mixture also magnetic supports retain their antibody binding capability and bound 117 mg IgG per g of support. The purity of elution's from magnetic nanoparticles were analysed by SDS–PAGE (figure 5.10). After confirmation by SDS-PAGE electrophoresis of the binding and elution capacity of the support on the crude extract used, the samples collected were quantified by BCA method in order to determine the amount of protein bound to and eluted from the support.

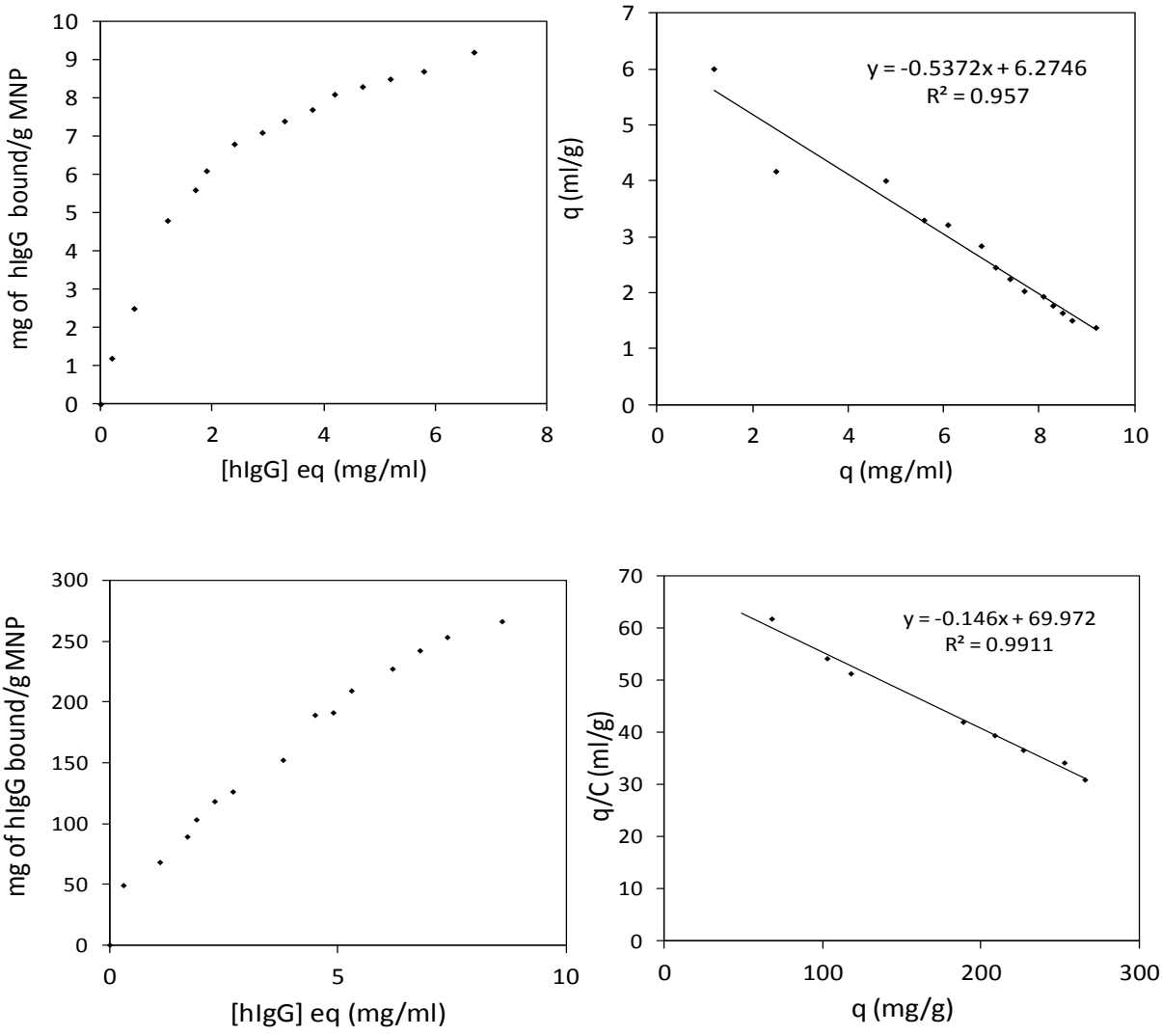


Figure. 5.10 Binding of human IgG at the surface of MP-A2C7I1 (A; B) and MP-DX-OX Control (C; D). Representation of q (the amount of bound hIgG in equilibrium per mass of solid support) as function of C_{eq} (the concentration of hIgG in equilibrium). Experimental data was fitted with the expression $q = (Q_{max} \times C_{eq}) / (K_d + C_{eq})$ using Scatchad plot, where Q_{max} corresponds to the maximum concentration of the matrix sites available to the partitioning solute (which can also be defined as the binding capacity of the adsorbent), and K_d is the dissociation constant. Maximum concentration of the matrix sites available to the partitioning solute.

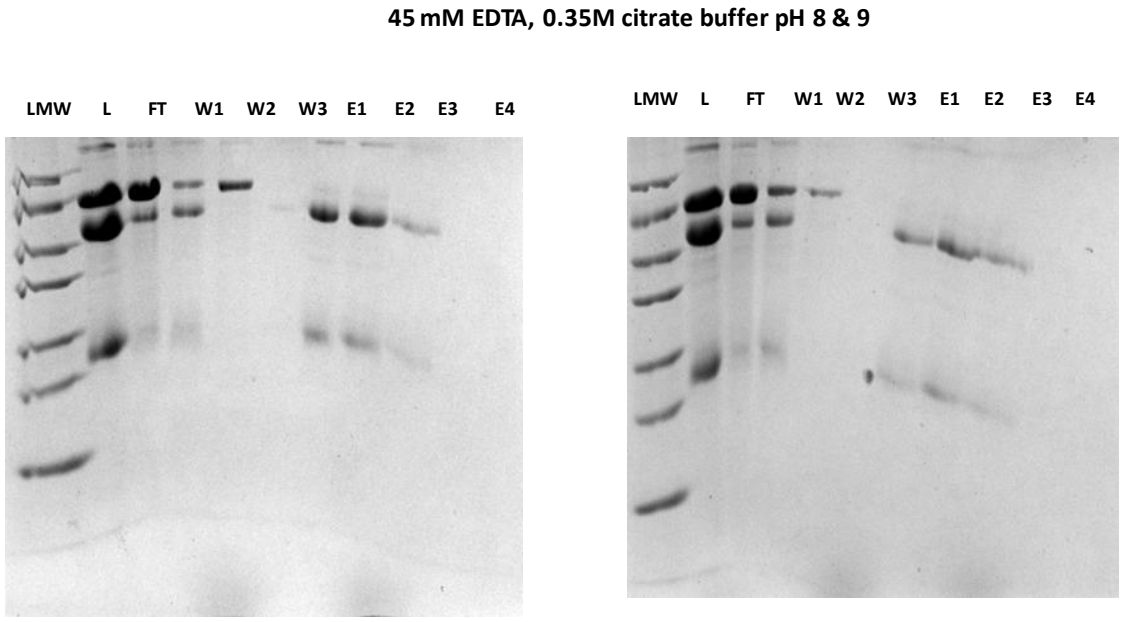


Figure 5.11 Electrophoreses gel in denaturation conditions to verify the binding capacity as well as the best elution conditions for IgG from the oxidized dextran coated MPs modified with ligand A2C7I1 by direct magnetic separation. LMW (Low Molecular Weight); Load (Loading Sample of the crude extract incubated with the adsorbent); W1 (First Wash with Binding Buffer-50mM Phosphate buffer of pH-8); E1 (First Elution-50mM Glycine-NaOH buffer of pH-11); E2 (Second Elution); D-W1 (First wash with Binding Buffer-50mM Phosphate buffer by direct method); D-E1 (First wash with Elution Buffer by direct method); D-E2 (Second wash with Elution buffer by direct method).

5.7 Protein binding of ligand A2C7I1 modified using glutaraldehyde method

MP-Glut-A2C7I1 were tested with BCA test before and after the ligand modification. The results are showed in Figure 5.11.

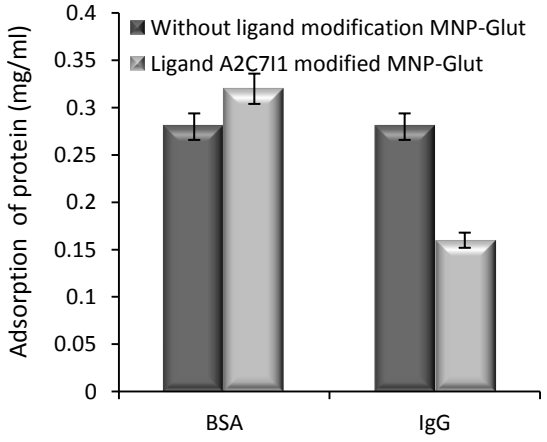


Figure 5.12. Adsorption of BSA and IgG for A2C7I1 modified MNP-Glut

Particles functionalized with glutaraldehyde and modified with ligand A2C7I1 adsorbed 0.16 mg of IgG/ml and the same particles but without ligand modification adsorbed 0.28 mg of IgG/ml. However, particles functionalized with glutaraldehyde and modified with ligand A2C7I1 showed adsorption of 0.28 g of BSA/ml and MP-Glut without ligand modification adsorbed 0.32 g of BSA/ml. Therefore, ligand A2C7I1 modification with this type of functionalized particles does not showed improvement of specific protein binding. Thus, more tests are required to improve this ligand modification.

5.8 Optimization of buffer solution for effective IgG elution

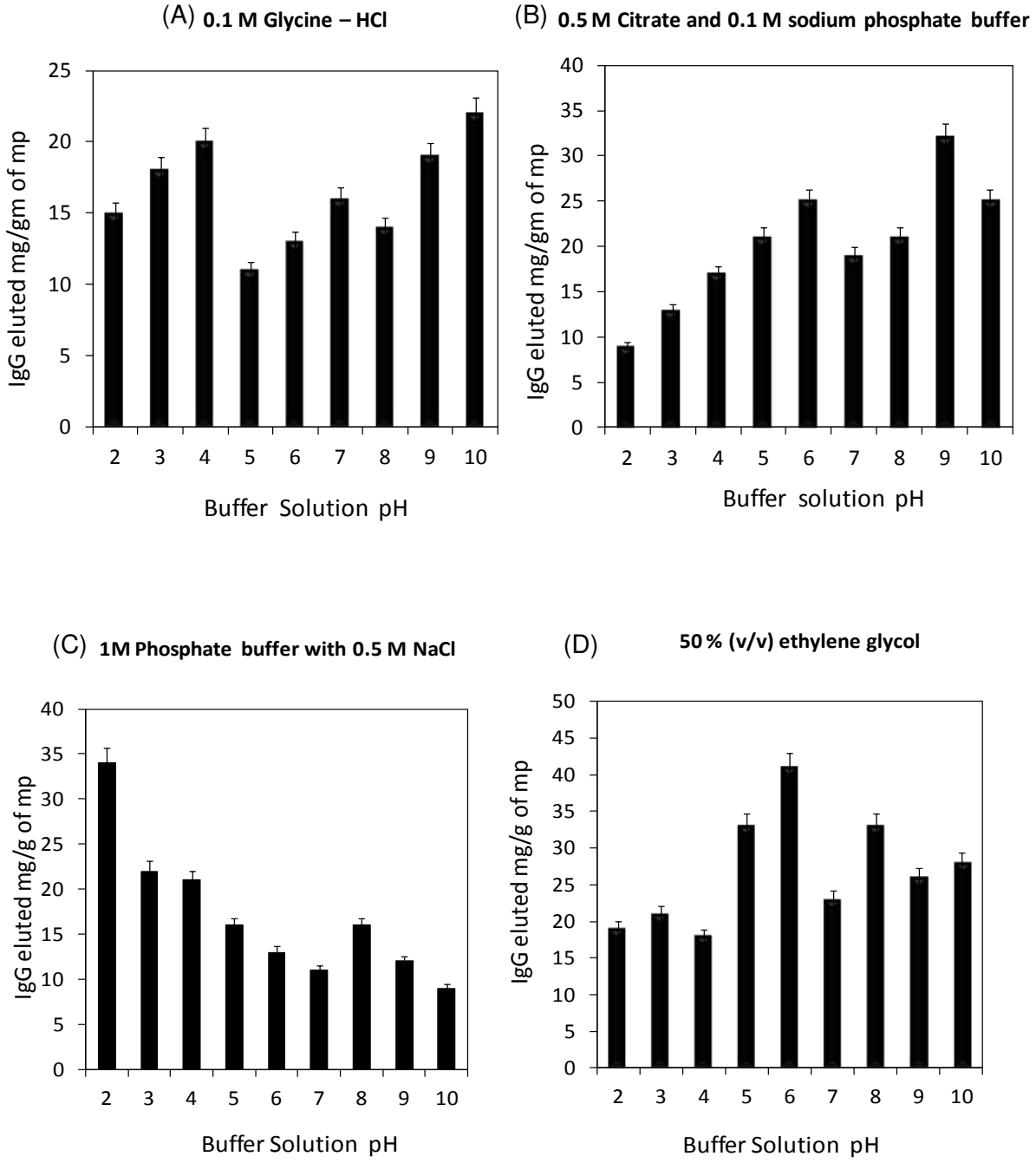


Figure 5.13 Percentage of hIgG eluted from MP-DX-A2C7I1 using various buffers at different pH conditions.(A) 0.1 M Glycine-HCl, (B) 0.5 M Citrate and 0.1 M Sodium phosphate buffer,(C) 1 M Phosphate buffer with 0.5 M NaCl,(D) 50% (v/v) ethylene glycol

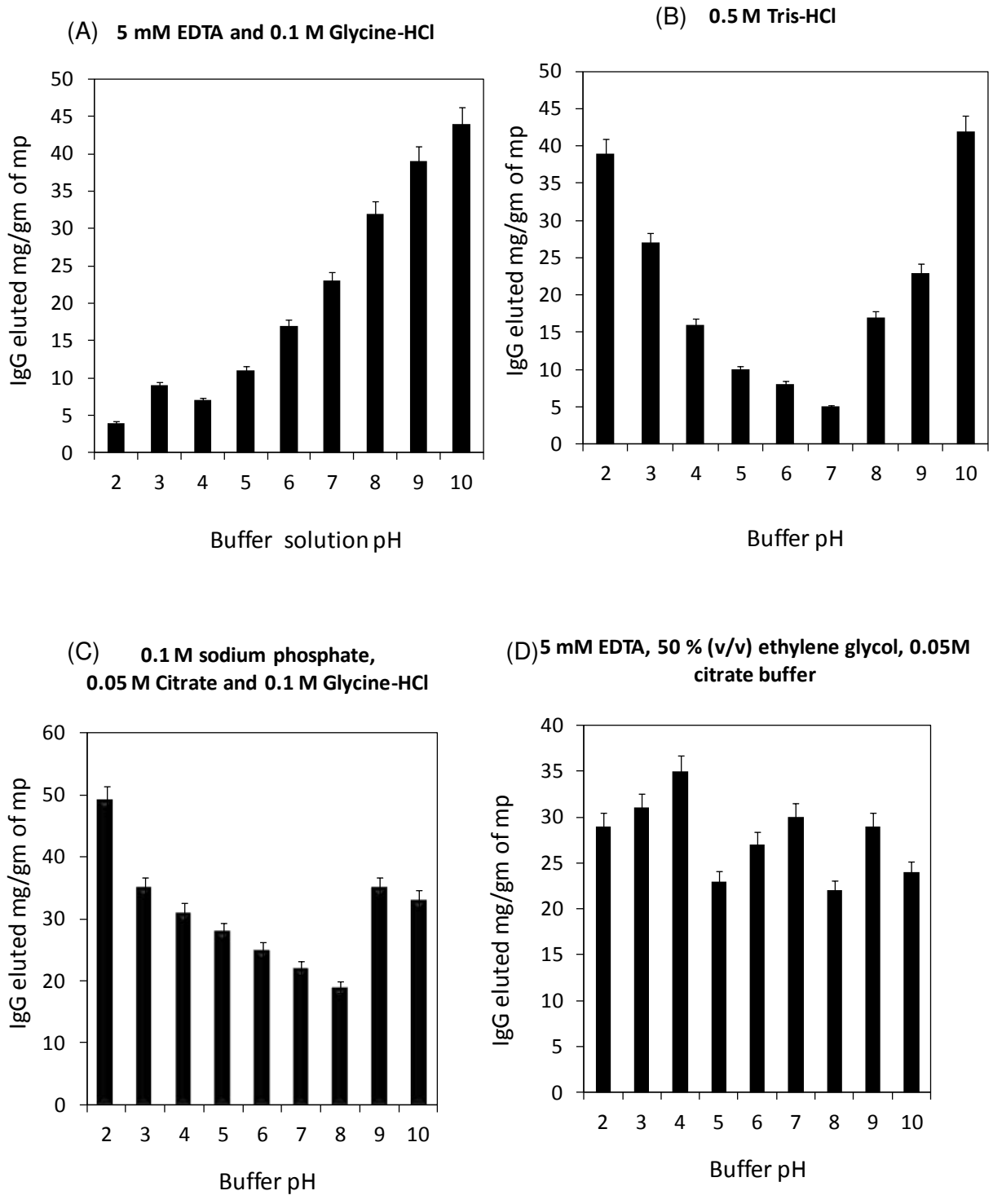


Figure 5.14 Percentage of hIgG eluted from MP-DX-A2C7I1 using various buffers at different pH conditions. 5 mM EDTA and 0.1 M Glycine-HCl (B) 0.5 M Tris-HCl (C) 0.1 M Sodium Phosphate, 0.05 M citrate and 0.1 M Glycine-HCl (D) 5 mM EDTA, 50 % (v/v) ethylene glycol, 0.05 M Citrate buffer

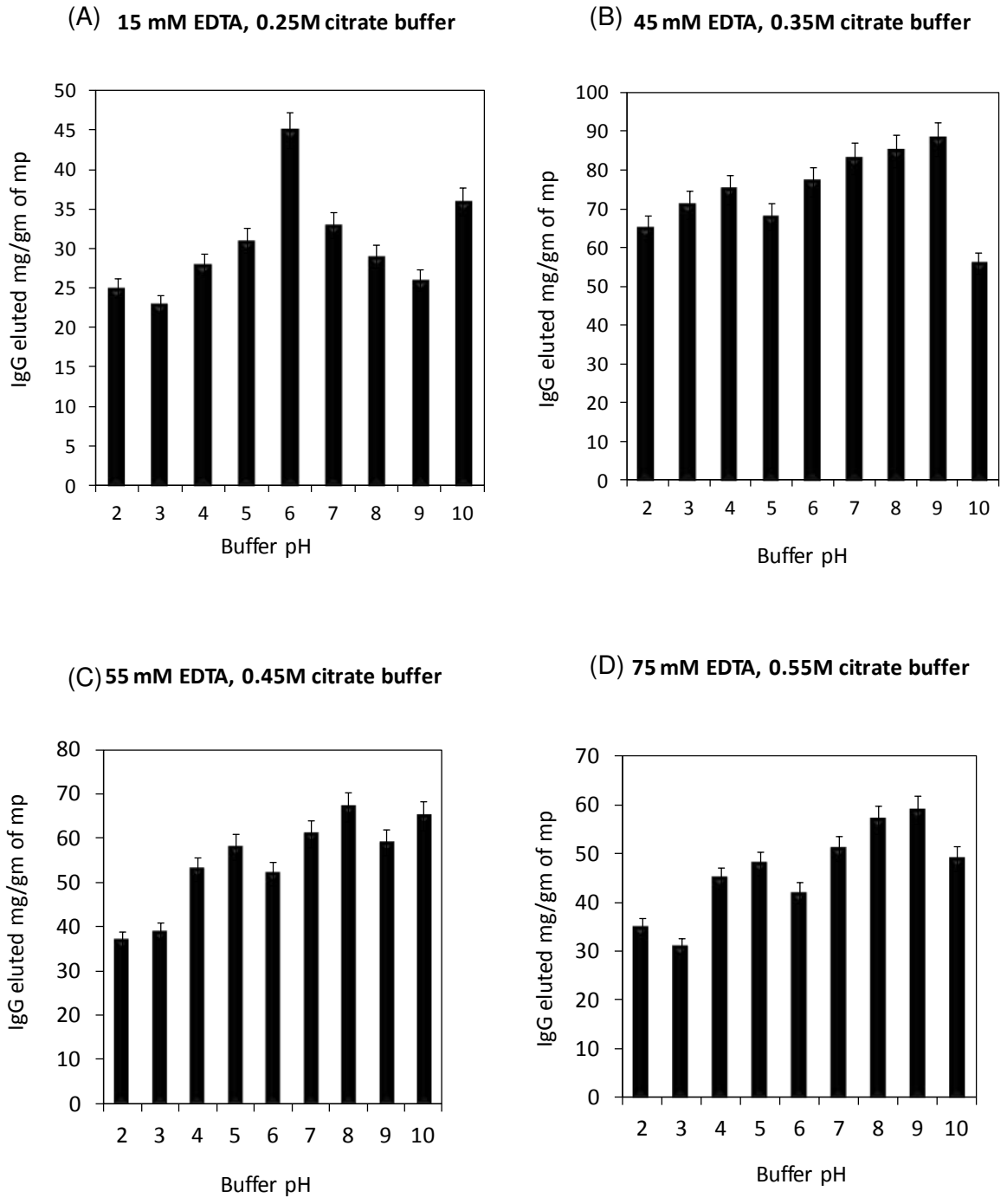


Figure 5.15 Percentage of hIgG eluted from MP-DX-A2C7I1 using various buffers at different pH conditions. (A) 15 mM EDTA, 0.25 M Citrate buffer (B) 45 mM EDTA, 0.35M citrate buffer (C) 55 mM EDTA, 0.45M citrate buffer (D) 75 mM EDTA, 0.55 M citrate buffer

5.9 Conclusion

The particles synthesized using two coprecipitation method and modified with Ligand A2C7I1 using Ugi reaction shows better applicability for magnetic separation. The particles synthesized using co-precipitation method show higher adsorption of dextran as compared to particles synthesized by solvothermal method. Also, solvothermal method is best to have micro size particles, whereas co-precipitation method is best to synthesize nano size particles. By comparing the cost of MP production by solvothermal and co-precipitation method, it is observed that the cost of production is 20, 87 euros per gram of MPs and 0,687 euros per gram of MP, respectively.

Coating and functionalization was performed in MP produced by solvothermal method. Particles coated with silica followed by dextran coating were able to adsorb the maximum amount of dextran: 2.2 mg dextran/g of MP observed at high concentration. MP particles able to provide amination value of 358 µmol/g of MP. When contacted with pure protein solution of Bovine Serum Albumin (BSA), the particles bound 3.85 mg/g of MP. In the characterization of MPs by DLS, the hydrodynamic diameter of coated particles is greater than bare particles. Silica coating and dextran coating during the reaction showed the results with most higher diameter, reaching 4000 nm 8000 nm. The zeta-potential decreases with dextran coating due to neutral nature, however silica-coated MPs has a zeta potential value at the same range than bare MPs. By VSM, it was possible to conclude that all types of particles showed superparamagnetic properties.

Particles were functionalized with dextran oxidized and glutaraldehyde and a ligand modification was performed based on Ugi Reaction. Ligand A2C7I1 was tested and the non-apecific binding of particles were reduced when particles were functionalized with oxidized dextran. More studies are required to optimize the ligand modification and biopurification.

References:

[1] Bruce, I. J. and Sen, T. (2005). Surface Modification of Magnetic Nanoparticles with alkoxysilanes and their Application in Magnetic Bioseparations.Langmuir 21: 7029-7035.

[2] Cakmak, S., Guemuesderelioglu, M. and Denizli, A. (2009). Biofunctionalization of magnetic poly (glycidyl methacrylate) micro spheres with protein A : Characterization and cellular interactions.Reactive & Functional Polymers 69(8): 586-593.

[3] Dhadge V.L., Hussain A., Azevedo A., Aires-Barros M. R., Roque A.C.A., Boronic acid modified magnetic materials for antibody purification, J. R. Soc. Interface 11 (2013) 20130875.

[4] Dhadge V.L., Azevedo A., Aires-Barros M. R., Roque A.C.A., Magnetic Aqueous Two Phase Fishing : An hybrid process technology for antibody purification, Submitted to Journal of Chromatography A.

[5] Ditsch, A., Laibinis, P. E., Wang, D. I. C. and Hatton, T. A. (2005). Controlled Clustering and Enhanced Stability of Polymer-Coated Magnetic Nanoparticles. Langmuir 21(13): 6006-6018.

[6] Borlido L., Azevedo AM, Sousa AG, Oliveira PH, Roque ACA, Aires - Barros MR.,Fishing human monoclonal antibodies from a CHO cell supernatant with boronic acid magnetic particles. Journal of Chromatography B, 2012. 903: p. 163-170.

[7] Grillo,M. E.,Finnis, M.W. Ranke, W. (2008). Surface structure and water adsorption on Fe_3O_4: Spin-density functional theory and on-site Coulomb interactions. Physical ReviewB77:075407.

[8] Haigh, J. M., Hussain, A., Mimmack, M. L. and Lowe , C. R. (2009). Affinity ligands for immunoglobulins based on the multicomponent Ugi reaction. J of Chromatography B877:1440–1452.

[9] Hung, C.-W., Holoman, T. R. P., Kofinas, P. and Bentley, W. E. (2008). Towards oriented assembly of poteins onto magnetic nanoparticles. Biochem. Engineering Journal 38: 164–170.

[10] JianHan, X., Hui, S., Wei, Z., Yi, L., JiaXin, D. and Günter, B. (2007). Surface chemistry of nanoscale Fe3O4 dispersed in magnetic fluids. Science in China Series B: Chemistry 50(6): 754-758.

[11] Konwarh, R., Karak, N., Rai, S. K. and Mukherjee, A. K. (2009). Polymer-assisted iron oxide magnetic nanoparticle immobilized keratinase. Nanotechnology 20(22).

[12] Kumar, M. K., Reddy, A. L. M. and Ramaprabhu, S. (2008). Exfoliated single-walled carbon nanotube-based hydrogen sensor.Sensors and Actuators B 130: 653–660.

[13] Lattuada,M.and Hatton,T. A.(2007).Functionalization of Monodisperse Magnetic nanoparticles. Langmuir 23: 2158-2168.

[14] Laurent, S., Forge, D., Port, M., Roch, A., Robic, C., Elst, L. V. and Muller, R. N. (2008). Magnetic Iron Oxide Nanoparticle : Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications. Chemical Reviews 108: 2064–2110.

[15] Li, J. and Gao, H. (2008). A Renewable Potentiometric Immunosensor Based on Fe3O4 Nanoparticles Immobilized Anti-IgG. Electroanalysis 20(8): 881-887.

[16] Liang,Y.Y., Zhang, L.-M., Li, W. and Chen, R.-F. (2007). Polysaccharide-modified iron oxide nanoparticles as an effective magnetic affinity adsorbent for bovine serum albumin. Colloid Polym Sci 285: 1193–1199.

[17] Lu, A., Salabas, E. L. and Schüth, F. (2008). Magnetic Nanoparticles: Synthesis, Protection, Functionalization, and Application. Angewandte Chemie International Edition 46: 1222 – 1244.

[18] Park, C., Park, K. H., Kwon, D. and Yoon, T. H. (2008). Biocompatible Quantum Dot Nanocolloids Stabilized by Gum Arabic. Bulletin of the Korean Chemical Society 29(6): 1277-1279.

[19] Roque,A.CA, Bicho A, Batalha IL, Cardoso AS, Hussain A. (2009). Biocompatible and bioactive gum arabic coated iron oxide magnetic nanoparticles. Journal of biotechnology. 144 (4): 313-320.

[20] Santana D. F., Dhadge V. L., Roque , A.C.A ., Dextran coated magnetic supports for antibody purification. ACS Appl. Mater. Interfaces, 2012. 4(11): p. 5907-5914.

[21] Cruz, M., Freitas, F., Torres, C.A.V., Reis, M.A.M., and Alves, V.D. Influence of temperature on the rheological behavior of a new fucose-containing bacterial exopolysaccharide. International Journal of Biological Macromolecules.48, 695-699, 2011.

[22] Torres, C.A.V., Marques, R., Antunes, S., Alves, V.D., Sousa, I., Ramos, A.M., Oliveira, R., Freitas, F., and Reis, M.A.M. Kinetics of production and characterization of the fucose-containing exopolysaccharide from enterobactor A47. Journal of biotechnology. 156, 261-267, 2011.

[23] Freitas, F., Alves, V.D., Pais, J., Costa, N., Oliveira, C., Mafra, L., Hilliou, L., Oliveira, R., and Reis, M.A.M. Characterization of an extracellular polysaccharide produced by a Pseudomonas strain grown on glycerol. Bioresource technology.100, 859-865, 2009.

[24] Freitas, F., Alves, V.D., and Reis, M.A.M. Advances in bacterial exopolysaccharides: from production to biotechnological applications. Trends in biotechnology.29:8, 388-397, 2011a.

[25] Freitas, F., Alves, V.D., Torres, C.A.V., Cruz, M., Sousa, I., Melo, M.J., Ramos, A.M., and Reis, M.A.M. Fucose-containing exopolysaccharide produced by the newly isolated Enterobacter strain A47 DSM 23139. Carbohydrate polymers.83, 159-165, 2011b.

[26] Cimini, D., De Rosa, M., and Schiraldi, C. Production of glucuronic acid-based polysaccharides by microbial fermentation for biomedical applications. Biotechnology Journal, 7:2, 237-250, 2012.

[27] Shamim, N., et al., , Adsorption, desorption, and conformational changes of lysozyme from thermosensitive nanomagnetic particles. Journal of Colloid and Interface Science, 2008. 320(1): p.15

[28] Adriaenssens E.M., L. S. M., Vandersteegen K., (2012). CIM® monolithic anion-exchange chromatography as a useful alternative to CsCl gradient purification of bacteriophage particles. Virology, 434(2), 265-270.

[29] Aguilar O. et. al. (2010). Coupled application of aqueous two-phase partitioning and 2D-electrophoresis for characterization of soyabean proteins. Sep. Sci. Technol., 45, 2210-2225.

[30] An X., S. Z., Zeng H.,. (2003). Preparation of highly magnetic chitosan particles and their use for affinity purification of enzymes. Journal of Chemical Technology & Biotechnology, 78(5), 596-600.

[31] Asenjo J.A. and Andrews B.A. (2011). Aqueous two-phase systems for protein separation: a perspective. J. Chromatogr. A, 1218(49), 8826-8835.

[32] Bandeira V., P. C., Rodrigues A.F., (2012). Downstream processing of lentiviral vectors: releasing bottlenecks. Hum. Gene Ther. Method, 23(4), 255-263.

[33] Becker, J. S., O.R.T. Thomas, and M. Franzreb. (2009). Protein separation with magnetic adsorbents in micellar aqueous two-phase systems. Sep. Purif. Technol., 65, 46-53.

[34] Bensch M., S. B., Hubbuch J., (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.

[35] Biao N., J. W., Xuejun C.(2009). Preparation and recycling of aqueous two-phase systems with pH-sensitive amphiphilic termopolymer PADB. Biotechnol. Prog., 25(3), 820-824.

[36] Chen S. et. al. (2009). A screening paradigm for the design of improved polymer-coated superparamagnetic iron oxide nanoparticles. . Journal of Materials Chemistry, 19(35).

[37] Ditsch A. et. al. (2005). High-Gradient Magnetic Separation of Magnetic Nanoclusters. Industrial & Engineering Chemistry Research, 44(17), 6824-6836.

[38] Goloverda G. et. al. (2009). Synthesis of ultrasmall magnetic iron oxide nanoparticles and study of their colloid and surface chemistry. . Journal of Magnetism and Magnetic Materials, 321(10), 1372-1376.

[39] Heebol-Nielsen A., J. S., Hobley TJ.,Thomas ORT.,(2004). Superparamagnetic cation-exchange adsorbents for bioproducts recovery from crude process liquors by high gradient magnetic fishing. Sep Sci Technol, 39, 2891-2914.

CHAPTER 6

HIGH GRADIENT MAGNETIC SEPARATION OF MONOCLONAL ANTIBODIES (MAbs)

SUMMARY

Magnetic materials synthesized as per method outlined in previous chapters were studied for suitability for use in high gradient magnetic separator. High gradient magnetic separator initially studied for development of magnetic field with application of various voltage strengths. Magnetic particles having boronic acid modified surface, particles with extracellular polysaccharide along with ligand 22/8 and ligand A2C7I1 were utilised in the HGMS setup to understand suitability for separation and purification of antibodies.

6.1 Introduction

The commonly used magnetic separation processes so far used were magnetocollection in which materials showing magnetic properties were passed over a magnet and collected where as the materials that were nonmagnetic pass out of magnetic field [1-3]. The industries that use magnetocollection widely was mineral processing industry [4] to recover iron ore as well as various process streams in which metallic materials removal was essential. In the case of larger particles magnetocollection works better but when particle size decreases magnetic separation becomes more and more difficult [5-7]. For smaller particles new method that attract attention of scientist and researchers is high gradient magnetic separation (HGMS) [8]. At larger scale HGMS find wide application in in Kaolin clay purification [9-11] in which removal of iron oxide improve the color of the clay, also steel industry utilize HGMS for fine metal particles removal [12-15]. Recently scientists find high gradient magnetic separation more convenient for water treatment and biologic extracts purification by magnetic seeding methods.

High gradient magnetic separator consists of central column fixed between the coils of electromagnet having stainless steel wire mesh packed into the column. As the wires are magnetisable high gradient magnetic field get developed near the wires where as open spaces between the wires remain non magnetic [16-19]. The magnetic nanoparticles that pass through the column of magnetic separator get attracted to the wires that were placed inside the column was represented by the following formula.

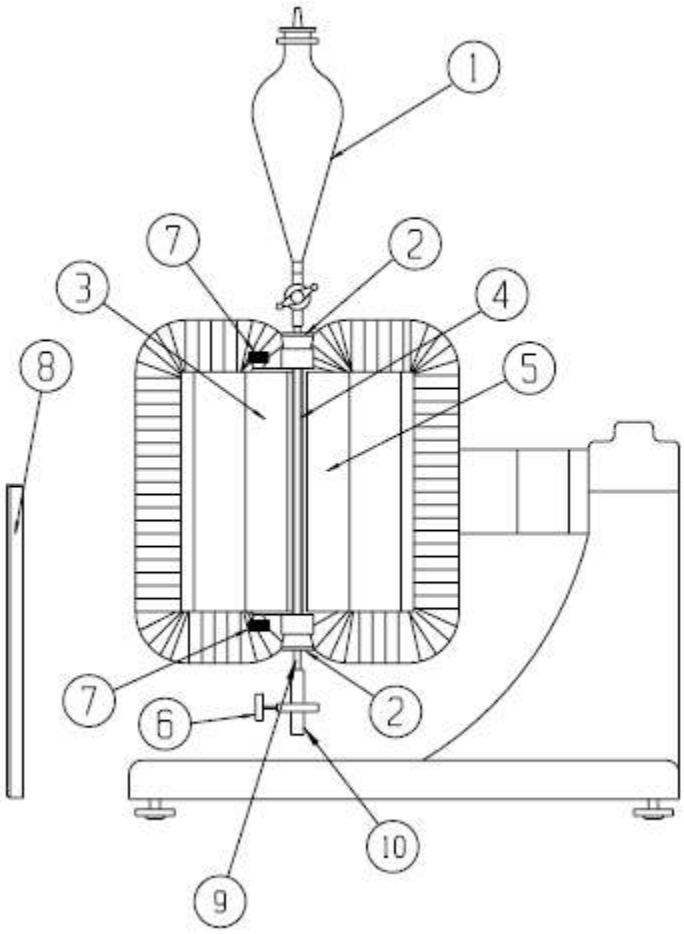
$$F_m = \mu_0 V_p M_p . \nabla H$$

Where μ_0 is the permeability of free space, V_p is the volume of the particle, Mp is the magnetization of the particle, and H is the magnetic field at the place of the particle. In magnetic particle based separations diffusion and equilibration occur in the order of milliseconds [20-22] and the magnetic capture step will be the rate limiting step in overall magnetic separations [23]. For effective separation the magnetic field at the place of particle must be large so that it overcome fluid drag as well as diffusion which will able to allow particle accumulation inside the column packing material [24-27]. Usually the void fraction inside the bed is high to allow colloidal solution to flow [28]. While utilization of HGMS for nanoparticles recovery diffusion plays important role and separation becomes more difficult for materials having particle size below 50 nm [29 – 31].

6.2 Experimental set-up for High Gradient Magnetic Separator

The equipment used in this experiment works in the gravity-feed mode. The magnetic separation column is supplied with about 5% volume packed (14.5 g) fine type 430 stainless steel wool. The column provides possibility to use different type of packing materials like stainless steel expanded metal to vary packing density to accommodate different particle sizes and fluid viscosities. The matrix may be removed with the Packing Rod and a new matrix inserted and uniformly packed with the same Packing Rod.

The external power supply provides linked regulation of voltage and amperage, to permit operation at a selected current level for extended periods. Regulation provides automatic increases in voltage as the resistance of the coils increases with heating [32-33], in order that the selected current will be maintained. At start-up, when the separator's coils are cold, with the regulated power supply controls set to supply maximum voltage (150 V. d. c.) and current, the maximum current drawn by the magnet is about 2.3 Amperes. Since voltage cannot be increased above 150 V., however, the current regulation feature of the power supply is inoperative at these settings.



1. Canister Funnel
2. Canister Rubber Stopper
3. Left Canister Pole Piece
4. Canister Chute
5. Right Canister Pole Piece
6. Canister Clamp
7. Short Screw Fiber Washer
8. Canister Packing Rod
9. Rigid Tubing
10. Flexible Tubing

Figure 6.1. Schematic view of high gradient magnetic separator (HGMS)

For practical purposes maximum current levels that can be maintained in ambient temperature in the 18° C.to 32° C range are about 1.8 A. for intermittent operation, (in which the current is turned off after an hour or so of operation to remove separated fractions) and about 1.7 A. for continuous operation over periods of eight hours or more.

During start up of equipment initially clamp turn off to pre-fill the system with liquid before beginning a separation. Otherwise air pockets may decrease the efficiency of the separation. The stopcock is turned off, and the slurry to be separated is poured into Funnel. Using current regulation setting of the current for the desired magnetic background field was carried out. After that the stopcock on the Funnel and the Clamp were open until the desired flow rate is achieved. Once the separation is complete, Clamp and power supply turn off, and flush the matrix with liquid poured into Funnel. The Column may need to be removed from the flow system to allow more complete flushing. In extreme cases the matrix may need to be removed for thorough cleaning. In order to obtain the same magnetic field as with the Column in place, 0.062 in. (1.59 mm) thick steel shims need to be placed between both pole pieces and their respective mounting surfaces on the cores.

Resistance of the coils of the Canister Separator (Model L-1CN) connected in series should be between 58 and 68 Ohms. The resistance should be measured across the two coil leads with the coil cord disconnected. To check the resistance of each coil, the coil needs to be separated from each other. It should be between 29 and 34 Ohms. With the voltage and amperage controls of the regulated power supply at maximum settings the direct current input to the separator should be approximately 2.3 amperes. The development of magnetic field inside the column of HGMS with change of current in amperes and voltage is illustrated in figure 6.2.

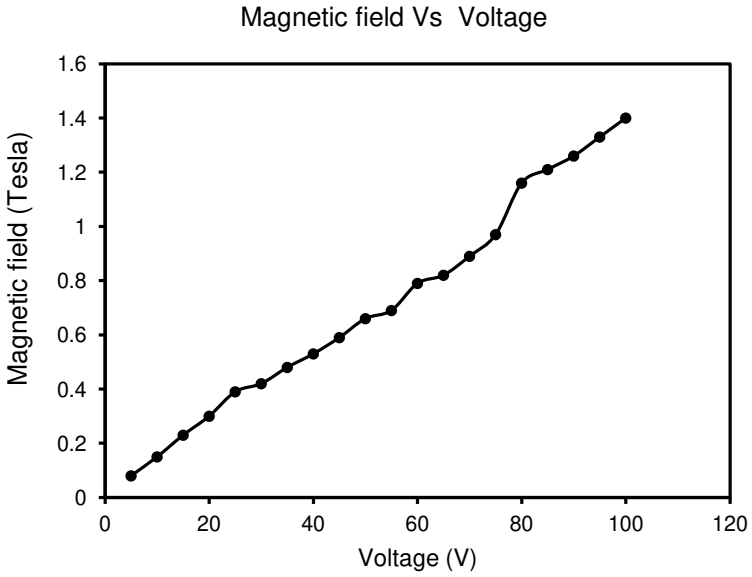


Figure 6.2. Magnetic field development inside the HGMS column in Tesla with increase in current in Volt

6.3 Boronic acid modified magnetic particles study in HGMS

The particles initially tested in above setup were modified with Glymo and then aminophenyl boronic acid. Aminophenyl boronic acids can form reversible covalent ester interactions with cis-diol-containing molecules, serving as a selective tool for binding glycoproteins as antibody molecules that possess oligosaccharides in both the Fv and Fc regions [34-36]. The particles used in this study, were first coated with dextran to avoid non-specific binding and then with 3-glycidyloxypropyl trimethoxysilane to allow further covalent coupling of APBA (APBA_MP).

The particles were characterized by DLS to determine the hydrodynamic diameter. As shown in figure 6.3 (A), the average hydrodynamic diameter increased from 255-270 nm to 700-720 nm, after dextran and APBA coating this size initially desired to be effective in HGMS but we still need further improvement in size in order to have superior application. Regarding the variation of zeta potential with different pH values, results shown in figure 6.3 (B) indicate that control particles have a zeta potential in the range of -5 mV at pH 3 varying gradually with the pH until reaching -9 mV at pH 11. In the case of boronic acid modified particles, the zeta potential variation with pH is very similar to the behaviour observed for the control particles between pH 3 and 8, being -6.3 mV at pH3. However, from pH 8 onwards, and particularly when shifting from pH 9 to 10, there is considerable drop in the zeta potential values (from -14mV to -23mV).This zeta potential variation found to be effective for operation in HGMS but still requirement is higher for more efficient use of HGMS equipment.

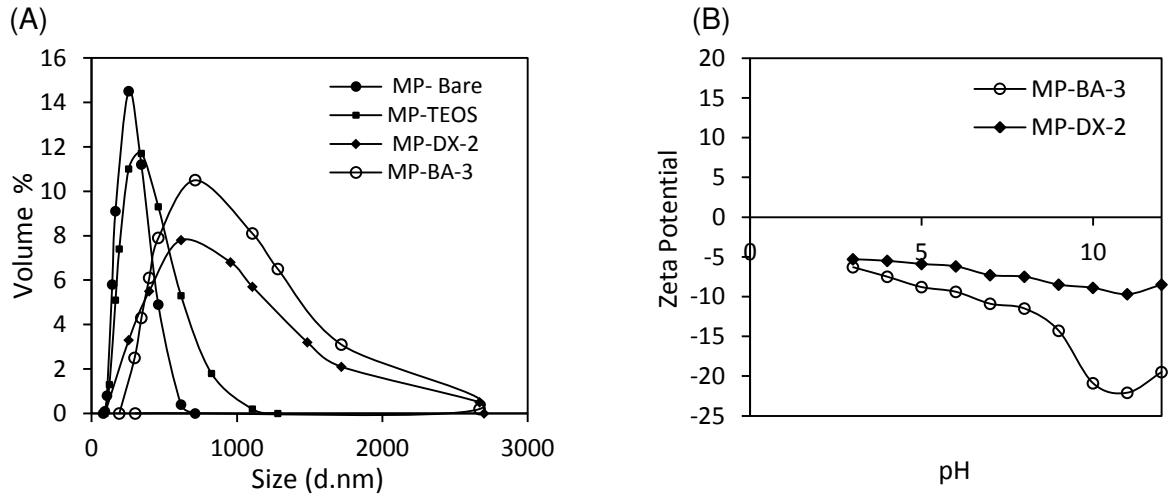


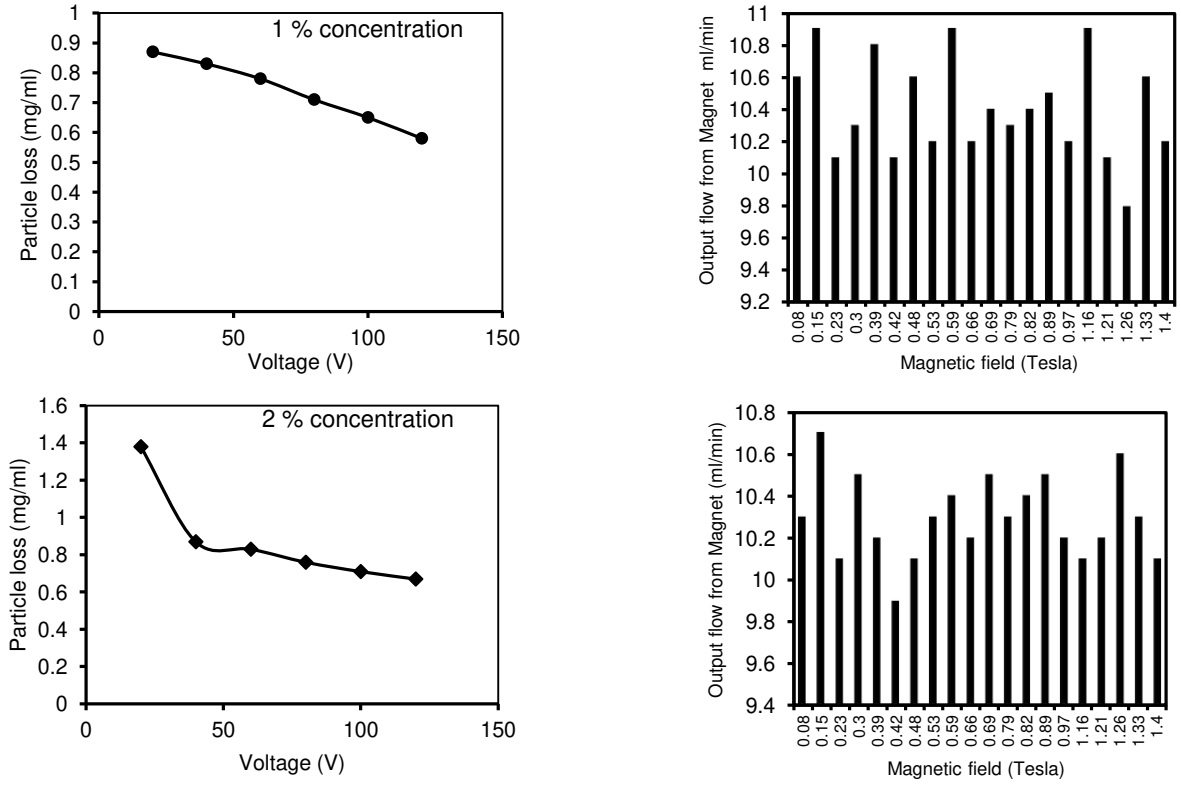
Figure 6.3(A) Size of magnetic particles at different coating and modification stages. 6.3(B) Changes in zeta potential of magnetic particles at different pH.

Eventhough, in terms of saturation magnetization, the values obtained were 37 emu/g for Bare-MP, and 34 emu/g for MP-BA-3, these particles shows less efficiency in binding to the inner wall of high gradient magnetic separation column and hence we need alternative particles which will have much bigger size as well as higher magnetic property to have more efficient and effective use of electromagnet.

In order to use the electromagnet at this particle condition we need to centrifuge and use permanent magnet to obtain maximum recovery of particles. As above particles testing in HGMS results in frequent losses and hence new particles having higher particle size is requirement.

6.4 HGMS performance at various concentration of Boronic acid coated magnetic particles

Various concentration of magnetic fluid prepared by dilution of boronic acid modified particles using Milli-Q water were studied to judge the capacity of electromagnet to handle maximum concentration of magnetic particles. Work was performed by removal of packing material inside the column. In the presence of packing material of stainless steel wire mesh it becomes difficult to remove all the magnetic particles after separation also it will consume more solvent (Milli-Q water) to again recover the particles. Initially desired concentrations of magnetic particles in the range of 1%, 2%, 3%, 4% and 5% were prepared. The particles along with Milli-Q water solution were then added in funnel of HGMS. This funnel directly fitted with agitator or externally fitted. In the case of external fitted agitator arrangement flow adjustment carried out using peristaltic pump. For direct agitation arrangement inside the funnel flow adjustment done using clamp fitted just below the funnel. Once the magnetic fluid charged inside the funnel, agitator started and voltage regulator switch on to pass the current to magnetic coils. Current that pass through the coils increased step by step in sequential manner in order to build up magnetic field inside the column. With gradual rise in voltage at specific interval samples of flow rate coming out of the column were collected and analysed for magnetic particle content that high gradient magnetic separator unable to capture.The losses from the HGMS at various voltage and magnetic field as well as output flow from the magnet are plotted in the figure 6.4.



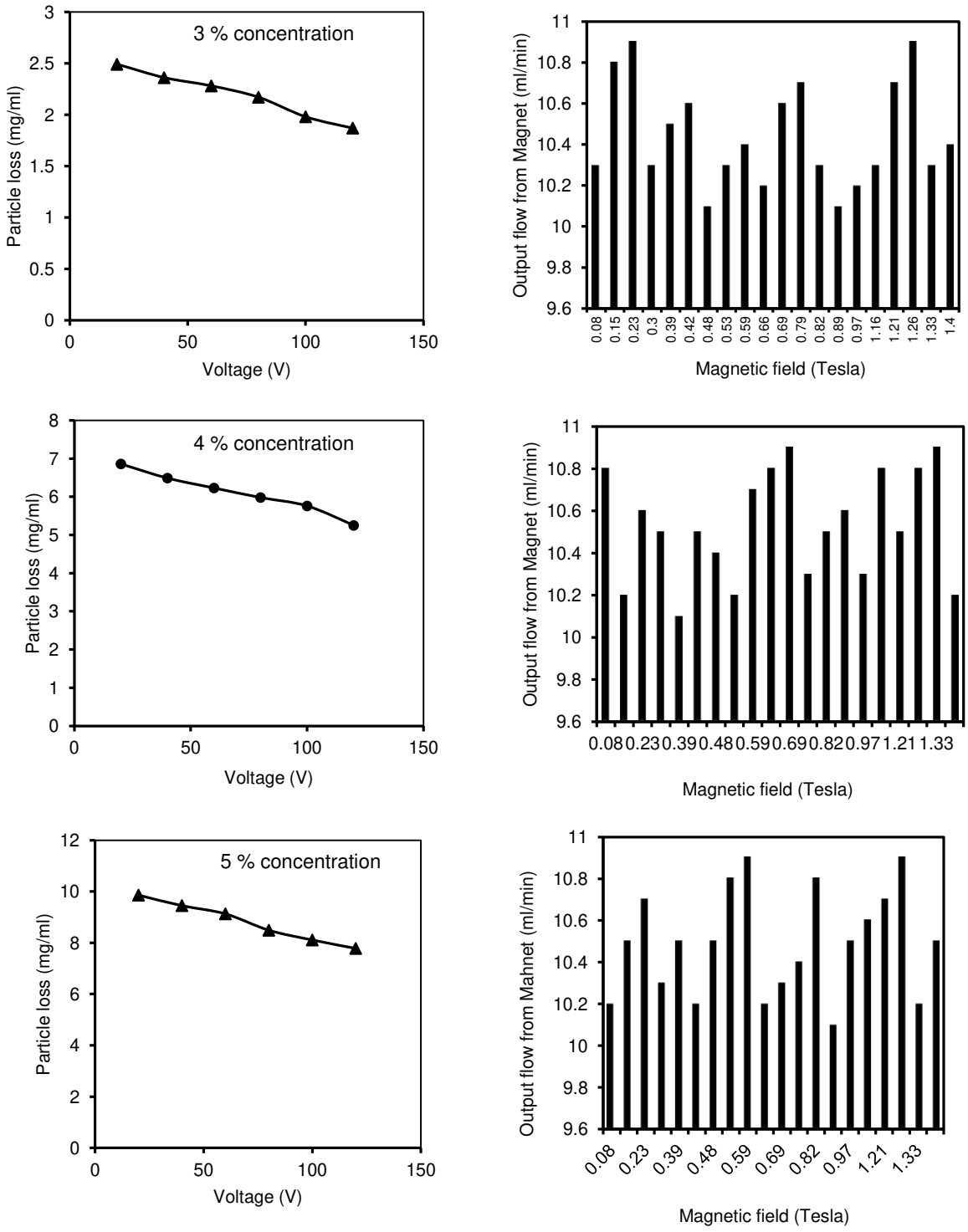


Figure 6.4 Boronic acid coated magnetic particle losses through HGMS at different voltage conditions and at various concentrations viz. 1 %, 2%, 3%, 4%, 5% with out put flow from magnet.

The gradual changes in flow rate that come out of the HGMS may be due to non-uniform particle size that enter column of HGMS. As some particles and their agglomerates settle due to gravity continuous agitation is preferred to have homogeneous flow of magnetic fluid to HGMS. Particle size and their shape well visualised and understand using TEM image as shown in figure 6.5.

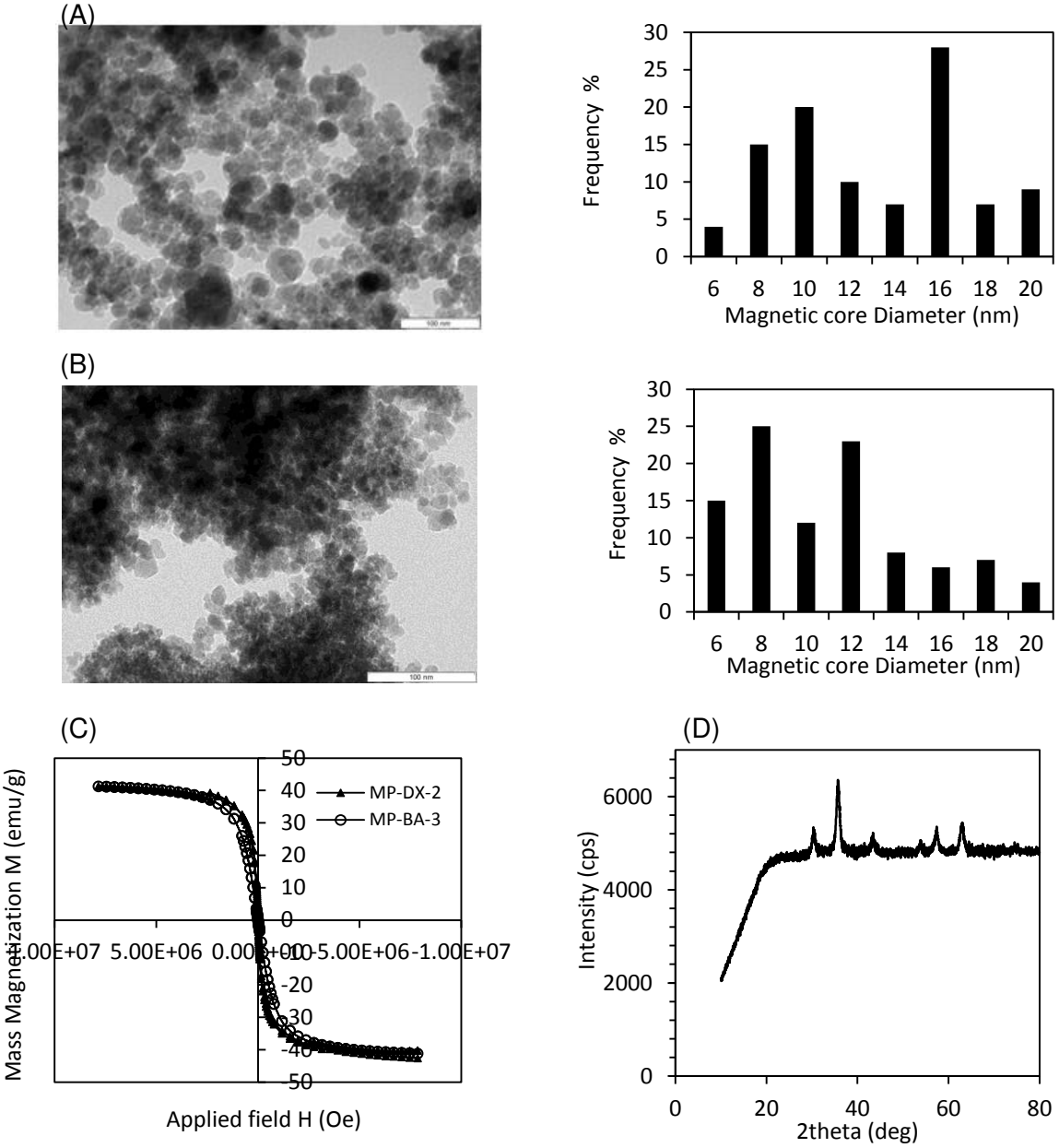
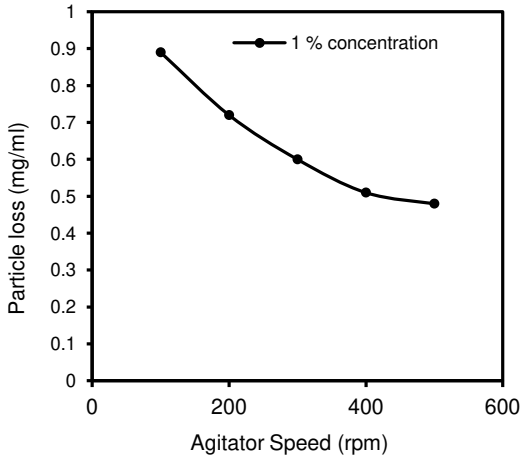
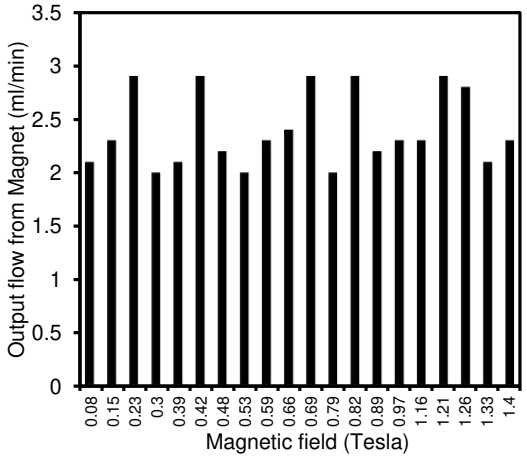
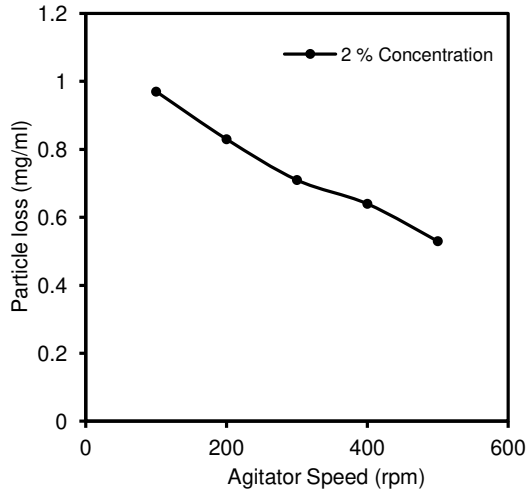


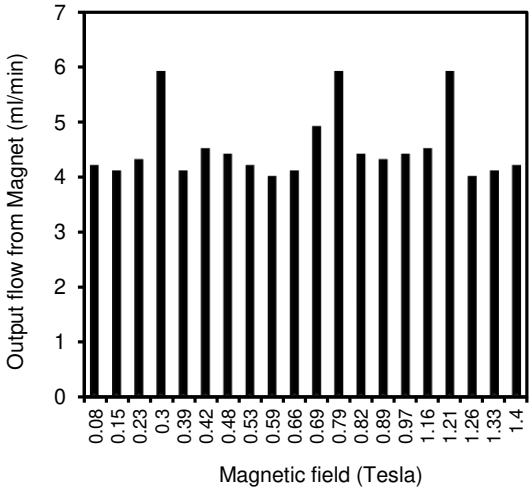
Figure 6.5 TEM images and magnetic properties of boronic acid magnetic particles used in HGMS study.

The characterization of particles by TEM confirmed the existence of spherical magnetic cores with an average diameter of 14 nm. Some heterogeneous population is also observed since the size distribution is between 6 – 20 nm (Figure A and B). With the VSM analysis it was possible to investigate the magnetic properties of the supports prepared. The curves shown in figure C represent a typical no hysteresis curve with reversibility and symmetry, characteristic of the superparamagnetic behavior of the particles synthesized. In terms of saturation magnetization, the values obtained were 37 emu/g for MP-DX-2, and 34 emu/g for MP-BA2. The saturation magnetization value obtained for the MPs are consistent with the values referenced in [37-40] for commercial aminophenol boronic acid coated MPs having the range of 35 emu/g. The XRD spectra represented in Figure D shows, five characteristic peaks for Fe_3O_4, marked by their indices ((2 2 0), (3 1 1), (4 0 0), (5 1 1), (4 4 0)). These peaks are consistent with database ICDD 2007 (International Centre for Diffraction Data) and revealed that the resultants MPs were Fe_3O_4.Particle losses with various agitator speed and output flow from the magnet were shown in the figure 6.6.









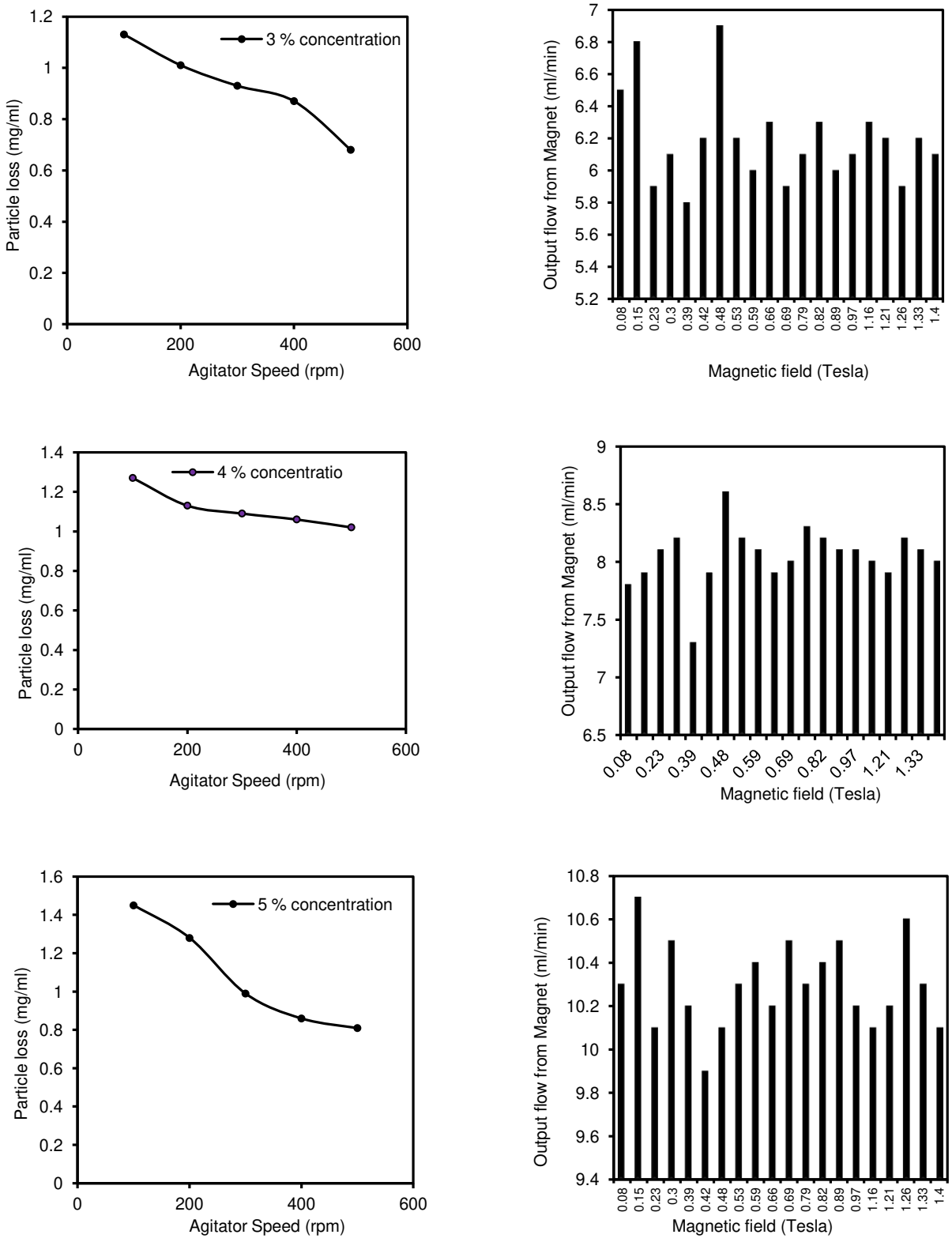


Figure 6.6 Boronic acid coated magnetic particle losses through HGMS at different agitator speed and at various concentrations viz. 1 %, 2%, 3%, 4%, 5% with out put flow from magnet.

6.5 HGMS study using pure antibody fractions

A 50 ml volume of boronic acid modified magnetic materials with a concentration of 10 mg/ml was washed sequentially with 50 ml regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and Milli-Q water and with elution buffer (1M Tris-HCl of pH 8.5) followed by 5 times washing with binding buffer (20 mM HEPES,pH-8.5). After that the supernatant was removed and resuspended in 50 ml IgG of concentration 1 mg/ml in binding buffer and incubated for 15 minutes at room temperature having agitation speed of 300 rpm. The mixture was then charged into HGMS funnel for separation. After addition of magnetic fluid electromagnet was then set into operation for magnetic field generation using magnetic coils. Magnetic fluid was allowed to pass through column for effective separation. Once the run get over magnetic particles were then recovered from the column washed with binding buffer and then eluted with elution buffer.All the washes were collected, centrifuged and separated in a magnetic support before being quantified by the BCA method.

After studying the supports for hIgG purification, the best supports were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The APBA functionalized magnetic materials were tested with the pure hIgG solution, by incubating for 15 minutes at room temperature. The liquid in which particles were suspended were again removed by HGMS and then washed two times with binding buffer (20 mM HEPES, pH_8.5). After washing, MPs were eluted five times using elution buffer (1M Tris,HCl, pH_8.5). All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

Time required for hIgG to adsorb on the surface of magnetic particles having functionalised APBA molecules on its surface for larger volume of 50 ml was studied in order to finalise exact incubation time to be provided to have optimum separation ability. In this study five different test samples of boronic acid modified magnetic particles (10 mg/ml concentration were incubated at room temperature with hIgG solution (1 mg/ml) for different time intervals mainly 5,10,15 and 20 minutes respectively. Incubation was followed by washing the particles binding buffer (20 mM HEPES, pH 8.5) and elution using elution buffer (1M Tris-HCl of pH 8.5), in order to know maximum protein that we able to elute. All washes were collected and quantified by BCA method.

After studying the magnetic supports with pure solutions of hIgG, the selected magnetic material were tested with CHO cell culture supernatants. 50 ml boronic acid coated magnetic particles were (10 mg/ml) incubated with 50 ml of the crude extract respectively, without any pre-treatment, for 15 minutes at room temperature. Once incubation was over the particles haaving adsorbed antibodies on its surface were then feed to high gradient magnetic separator for separation.Supernatants were collected and the particles were washed two times with 50 ml binding buffer (20 mM HEPES, pH 8.5). After washing, MPs were eluted using elution buffer (1M Tris-HCl of pH 8.5), in order to study the best elution conditions. All

collected samples were quantified by affinity chromatography using Akta purifier system from GE healthcare (Uppsala, Sweden), using porous protein A affinity column from applied biosystems (Foster City, CA, USA). The APBA coated magnetic particles at higher volumes shows IgG binding capacity up to 148 mg/g of MP. The elution samples were analysed BCA as well as Protein-A HPLC to quantify amount of antibodies. From the results it was observed that in direct magnetic separation process using complex crude mixture IgG recovery of 112 mg/g of MP was observed.

6.6 HGMS study of EPS – ligand 22/8 magnetic particles

Particle size plays important role in high gradient magnetic separation. The modifications using EPS polymer increases the hydrodynamic diameter of the particle. As shown in figure, the average hydrodynamic diameter for EPS Coated MPs is around 1100 nm. The average hydrodynamic diameter for bare MPs varies in the range of 260-265 nm. In case of particles coated with tetraethoxysilane rise in average hydrodynamic diameter observed to 365-375 nm. The MPs with 22/8 have average diameter in the range of 970-995 nm, even though some particles of size upto 3000 nm are also observed in the suspension.

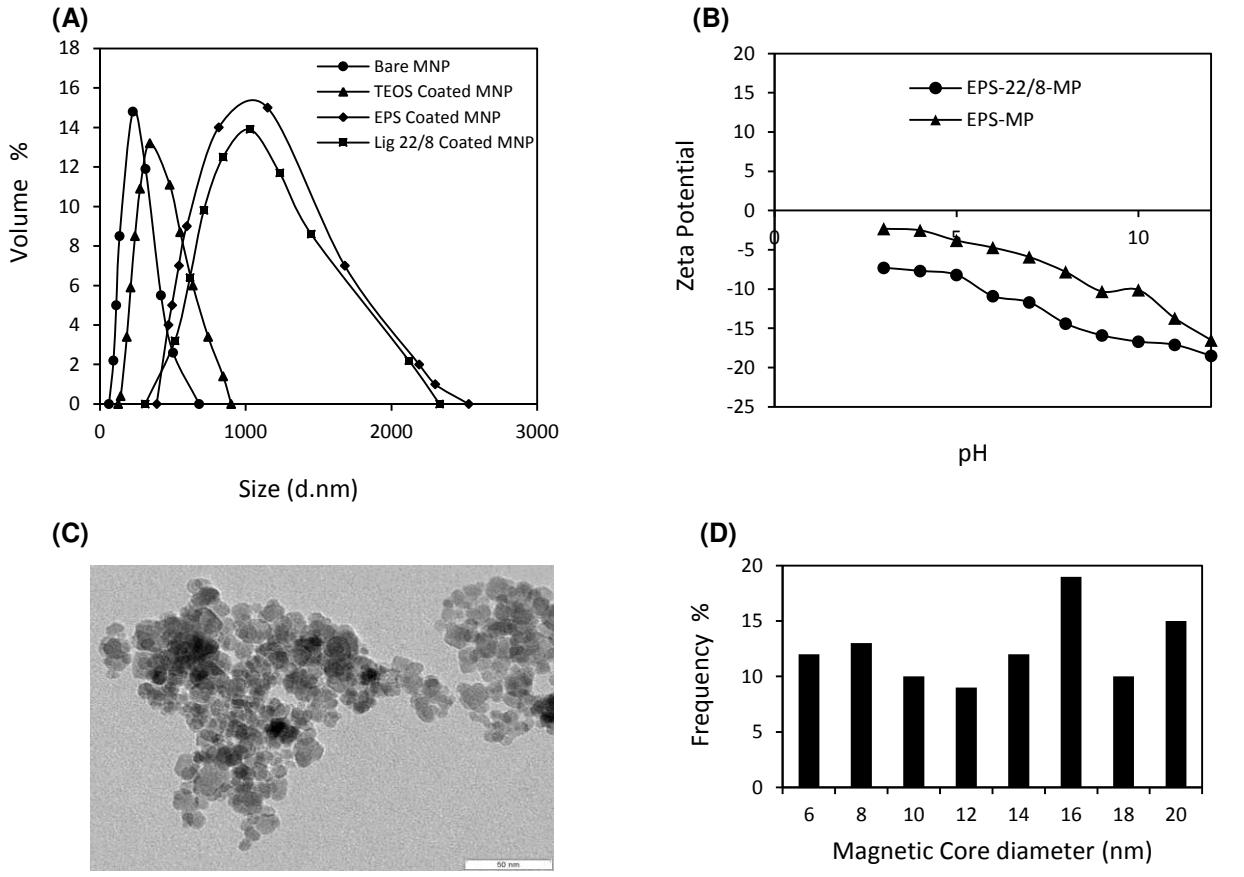


Figure 6.7. Physical properties and morphology of EPS-Ligand 22/8 coated magnetic particles used in HGMS study.

The morphology observation of particle using transmission electron microscopy shows formation of larger agglomerates when the particles were coated with polymers. It is observed that diameter of each individual magnetic core is in the range of 10-20 nm (figure-6.7, D). This might be explained by the non-covalent interactions between the coating biopolymers of neighbour nanoparticles. According to figure-6.7 C it is possible to conclude that the supports synthesized are not uniform in size and present a high polydispersitivity.

Through result data as shown in figure-6.7 B it was observed that zeta potential values of particle varies between -2 to -20 with variation of pH values between 3 to 12. EPS coated MPs have a zeta potential in the range of -2.5 to -17 which shows higher variation above pH 10. For EPS-22/8-MP at lower pH of 3 zeta potential value was -7.3 mV which increases gradually until pH 12 at which zeta potential value observed was -20.1.Usually, the values of zeta potential are attributed to the oxygen groups available in the tetrahedral and octahedral structure. When particles are coated with the different polymers the global charge is altered according to the type of biopolymer used. The magnetization values of both EPS-MP & EPS-22/8-MP varies between -60 e.m.u. per gm to + 60 e.m.u. per g following superparamagnetic behaviour.

The EPS-ligand 22/8 magnetic particles performance at various concentration with particle losses and flow rates are shown in following figures 6.8.

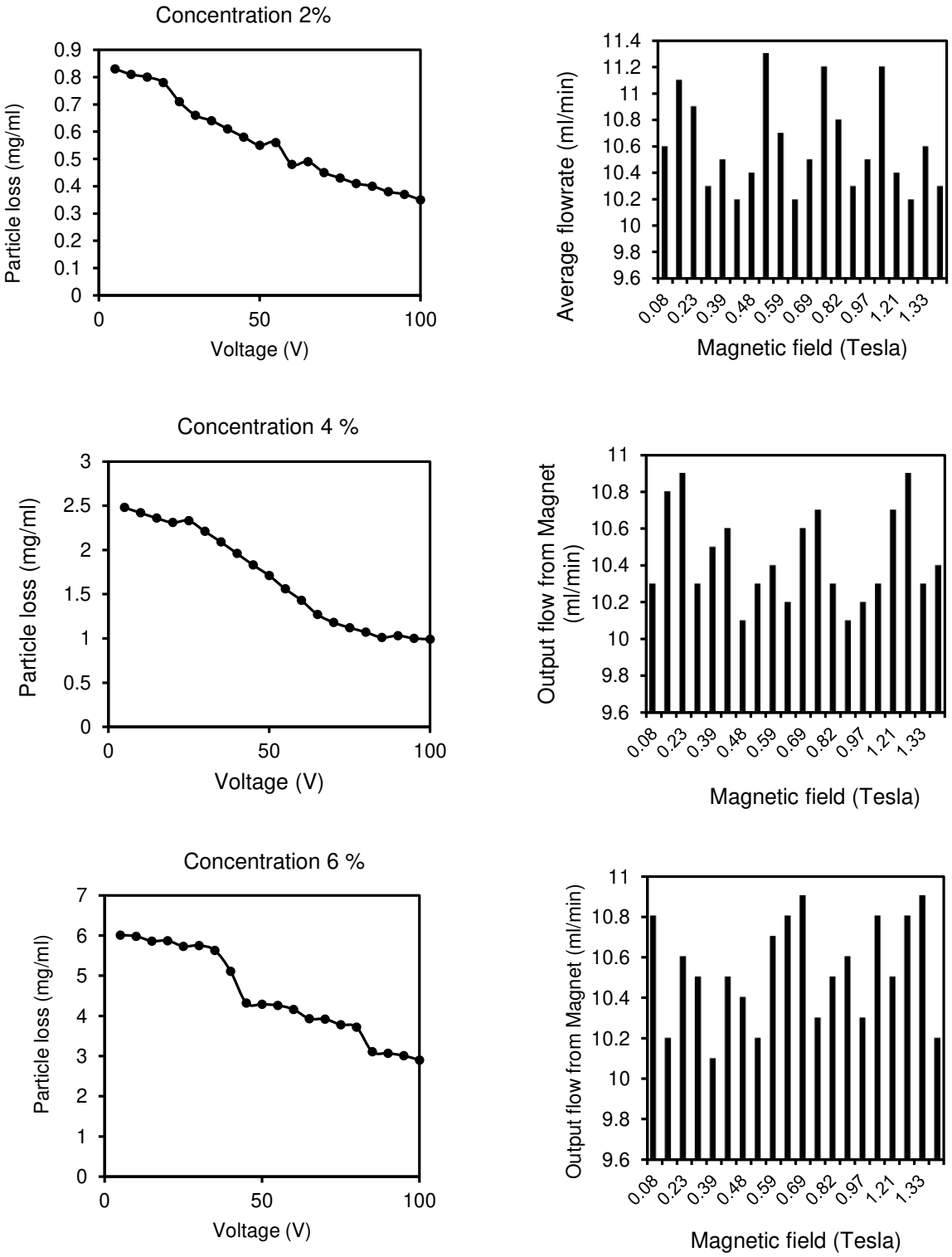
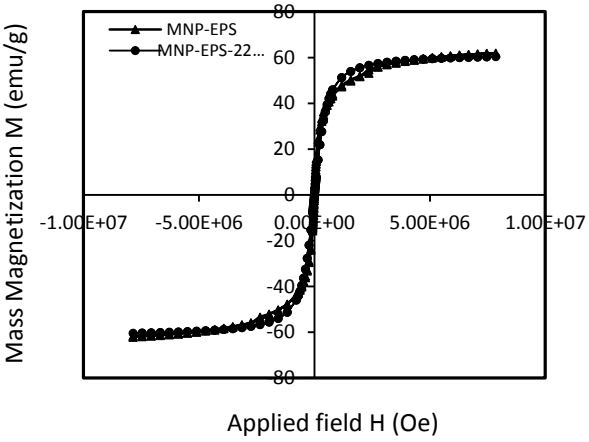


Figure 6.8 Ligand 22/8 coated magnetic particle losses through HGMS at different voltage conditions and at various concentrations viz. 2%, 4% and 6% with out put flow from magnet.

Eventhough the magnetization values of both EPS-MP & EPS-22/8-MP varies between -60 e.m.u. per gm to + 60 e.m.u. per g following superparamagnetic behaviour, losses of particles were observed from magnetic separator.



6.7 HGMS based Antibody purification using MP-EPS-22/8

The functionalized MP-EPS-22/8 were initially tested in HGMS with a pure solution of hIgG. The particles suspensions were washed firstly with 0.1 M NaOH prepared in 30% (v/v) isopropanol, followed by washing with deionized water. After washing the nanoparticles suspensions were equilibrated with 50 mM phosphate buffer of pH 8. After washing and equilibration of support the supernatants were removed and added with 50 ml of a hIgG solution of 1 mg/ml concentration diluted in 50 mM phosphate buffer. The incubation was carried out for 15 minutes at room temperature with constant stirring. The mixture was then charged to funnel of high gradient magnetic separator. supernatant was then collected and the particles recovered from the column were washed five times with 50 mL binding buffer. After preliminary washing steps elution of bound protein was then carried out using 50 mM Glycine – NaOH buffer of pH 11. All samples were analyzed by BCA assay with microplate reader in order to quantify the amount of protein bound to and eluted from the supports.

MP-EPS-22/8 were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The 22/8 functionalized (MP-EPS-22/8) (50 ml with 10 mg/ml) were tested with 50 ml of each of the pure hIgG solution, by incubating for 15 minutes at room temperature. The liquid in which particles were suspended were removed by magnetic separation and then washed two times with 50 ml binding buffer (50 mM Phosphate, pH-8). After washing, MPs were eluted five times using 50 mL elution buffer (50 mM Glycine-NaOH, pH-11). All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

Incubation time plays important role to adsorb antibody on the surface of magnetic particles. Higher volume of solution for HGMS study having functionalized MP-EPS-22/8 were studied in order to

finalise exact incubation time to be provided to have optimum separation ability. This study was carried out separately for direct magnetic separation. For direct magnetic separation incubation time studied with five different test samples of MP-EPS-22/8 having 50 ml volume were incubated at room temperature with 50 ml hIgG solution (1 mg/ml) for different time intervals mainly 5,8,10,12,14,16,18 and 20 minutes respectively. Incubation was followed by washing the particles two times with 50 ml binding buffer (50 mM Phosphate of pH 8) and five times elution using elution buffer (50 mM Glycine-NaOH of pH 11), in order to know maximum hIgG that we able to elute. All washes were collected and quantified by BCA method.

Magnetic supports MP-EPS-22/8 were tested with CHO cell culture supernatants in order to estimate efficiency of high gradient magnetic separator for crude purification. For direct magnetic separation technique 50 ml MP-EPS-22/8 with a concentration of 10 mg/ml were incubated with 50 ml of the crude extract, without any pre-treatment, for 20 minutes at room temperature. After incubation the particles were separated using HGMS and supernatants were collected. The separated particles were then washed five times with 50 ml binding buffer (50 mM Phosphate, pH 8). After washing, MPs were eluted using elution buffer (50 mM Glycine-NaOH of pH 11), in order to study the best elution conditions. All collected samples were quantified by affinity chromatography using Äkta purifier system from GE Healthcare (Uppsala, Sweden), using porous protein A affinity column from Applied Biosystems (Foster City, CA, USA). The BCA method and gel electrophoresis (12.5% Acrylamide/Bisacrylamide).

Bradford method was used to determine total protein concentration using a Coomassie reagent from Pierce (Rockford, IL, USA). The standard for protein calibration was made with bovine gamma globulin. Absorbance was measured at 595 nm in a Spectra Max 340PC microplate reader from Molecular Devices (Sunnyvale, CA, USA).

The MPs coated with 22/8 shows pure IgG binding up to 110 mg/g of MP. The elution samples were analysed BCA as well as Protein-A HPLC to quantify amount of antibodies. From the results it was observed that in direct magnetic separation process using complex crude mixture also magnetic supports retain their antibody binding capability and bound 92 mg IgG per g of support.

6.8 HGMS study of ligand A2C7I1 coated MNP

Based on Ugi Reaction, two types of MPs were tested, one functionalized with glutaraldehyde and other functionalized with oxidized dextran. The four-component reaction of synthesis process consists of reaction of MPs functionalized with aldehyde in methanol with an equimolar amount of 4-aminobenzamide in methanol, and the solution was left to stirr for 1 hour at room temperature. An equimolar amount of the carboxylic acid C7 (4-hydroxyphenylacetic acid) in methanol and isopropyl isocyanide were then added to the solution and left to stir for 48 hours at room temperature.

The Ugi reaction was first reported by Ivar Ugi in 1959 and it is a multi-component reaction in organic chemistry involving a ketone or aldehyde, an amine, an isocyanide and a carboxylic acid. The Ugi reaction has often been used as a tool in the synthesis of pharmacologically active molecules, natural products, therapeutic agents, and combinatorial libraries. The magnetic nanoparticles functionalized with oxidized dextran act as aldehyde molecule, due to the presence of aldehyde on the nanoparticle surface. For the Ugi reaction, the quantities of compounds were calculated on the basis of estimated aldehyde content using Sabolks method. The reagents used were 5 times excess the value obtained by analysis.To determine the concentration of ligands on MPs surface, the compounds that were used have sulfur in their composition. By Inductively Coupled Plasma Mass Spectrometry (ICP-MS), it is possible to determine the amount of sulfur in the solution, it follows that it is able to calculate the quantities of ligand.

For high gradient magnetic separator studies functionalized MP-A2C7I1 were first tested with a pure solution of hIgG. The particles suspensions were washed firstly with 0.1 M NaOH prepared in 30% (v/v) isopropanol, followed by washing with deionized water. After washing the nanoparticles suspensions were equilibrated with 50 mM phosphate buffer of pH 8. After washing and equilibration of support the supernatants were removed and added with 50 ml of a hIgG solution of 1 mg/ml concentration diluted in 50 mM phosphate buffer. The incubation was carried out for 20 minutes at room temperature with constant stirring. The mixture was then charged to high gradient magnetic separator for separation of magnetic particles and supernant. The supernatant was then collected and the particles were recovered from HGMS column which was followed by five times washing with 50 mL binding buffer. After preliminary washing steps elution of bound protein was then carried out using various buffer solutions of different pH range. All samples were analyzed by BCA assay with microplate reader in order to quantify the amount of protein bound to and eluted from the supports. In order to compare results the non-modified particles and ligand modified particles were tested at the same time and in the same conditions of the magnetic supports.

MP-A2C7I1 were tested for reuse applicability study with the purpose of testing the ability to reuse the support in purification processes. The A2C7I1 functionalized magnetic particles having concentration of 10 mg/ml were tested with 50 ml of each of the pure hIgG solution, by incubating for 20 minutes at room temperature. The liquid in which particles were suspended were removed by high gradient magnetic separation and then washed two times with 50 ml binding buffer. After washing, MPs

were eluted five times using 50 mL elution buffer. All eluted samples were analyzed using BCA method for hIgG quantification. In next step eluted MPs were regenerated using regeneration buffer (0.1 M NaOH in 30% (v/v) isopropanol) and again initial process step was repeated. In this way five cycles were carried out in order to know reuse suitability.

Incubation time plays important role to adsorb antibody on the surface of magnetic particles. Functionalized MP-A2C7I1 were studied in order to finalise exact incubation time to be provided for higher volume of HGMS to have optimum separation ability. For direct magnetic separation incubation time study five different test samples of MP-A2C7I1 having 50 ml volume were incubated at room temperature with 50 ml hIgG solution (1 mg/ml) for different time intervals mainly 5,8,10,12,14,16,18 and 20 minutes respectively. Incubation was followed by washing the particles two times with 50 ml binding buffer and five times elution using elution buffer, in order to know maximum hIgG that we able to elute. All washes were collected and quantified by BCA method.

6.9 High gradient magnetic separator Performance for handling A2C7I1 coated MPs

A2C7I1 coated particles were tested in High gradient magnetic separator at the concentration of 2 %, 4 %, and 6 %. The results are shown in figure 6.10. From results it is observed that for 2 % concentration particle loss at 100 volt is 0.3 mg/ml. Particle losses rise with concentration to 0.7 mg/ml and 1 mg/ml for 4 % and 6 % concentration.

The size distribuition of particles plays important role in High gradient magnetic separator. The size was measured by DLS, which is common analytical techniques and the associated range scale involved for nanoparticle sizing. The size of MPs is one of the parameter that often required due to many possible applications, because each application performs better with a particle having a certain range size [41-43]. The average size of particles without any type of coating was found to be 963 nm. The particles coated with dextran in situ while synthesis shows average particle size of 1530 nm. In case of particle coated with stirring shows slightly higher size than those coated in situ, average particle size in this case was in the range of 1830 nm. For silica coated particles there was sharp rise in particle size which shows average range of 1980 nm. From above results it was observed that coating increases the size of particle due to the formation of layer. Silica coating provides the larger increase and dextran-coated (in situ) MP has a higher size than MP coated with dextran after the reaction.

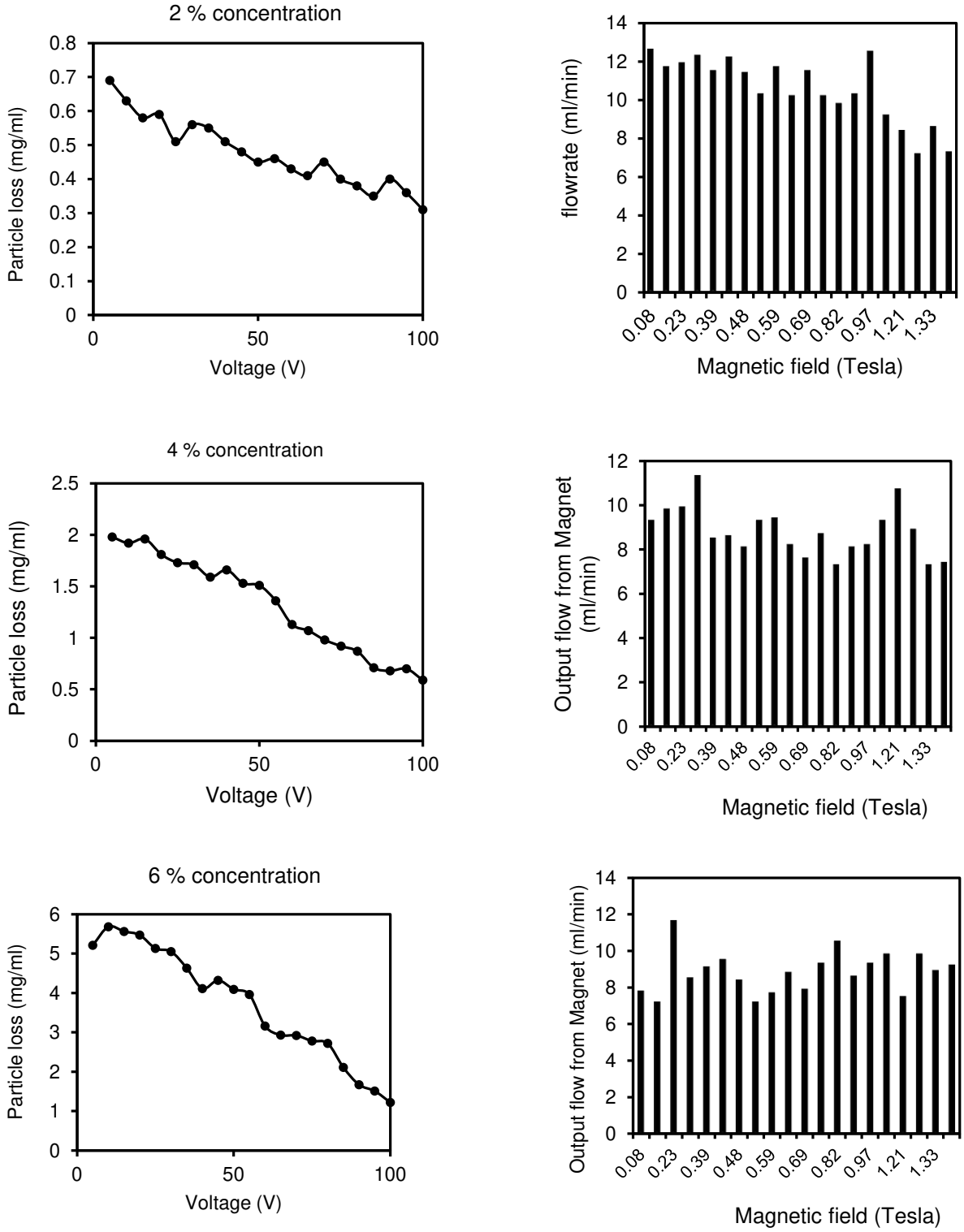


Figure 6.9 Ligand A2C7I1 coated magnetic particle losses through HGMS at different voltage conditions and at various concentrations viz. 2%, 4%, 6% with out put flow from magnet.

The size distribuition of particles plays important role in High gradient magnetic separator. The size was measured by DLS, which is common analytical techniques and the associated range scale involved for nanoparticle sizing. The size of MPs is one of the parameter that often required due to many possible applications, because each application performs better with a particle having a certain range size [41-43]. The average size of particles without any type of coating was found to be 963 nm. The particles coated with dextran in situ while synthesis shows average particle size of 1530 nm. In case of particle coated with stirring shows slightly higher size than those coated in situ, average particle size in this case was in the range of 1830 nm. For silica coated particles there was sharp rise in particle size which shows average range of 1980 nm. From above results it was observed that coating increases the size of particle due to the formation of layer. Silica coating provides the larger increase and dextran-coated (in situ) MP has a higher size than MP coated with dextran after the reaction.

In parallel, zeta potential was measured for all suspensions and determined approximate isoelectric points in order to determine the charge on the surface of particles. DLS was used to obtain these results. The average zeta potential of Bare MNP was -45.8 mV, this zeta reduces to near neutral level after coating with dextran which is a neutral polymer. In case of in situ dextran coating zeta potential was -0.0146 while in case of externally dextran coated particles zeta potential found to be -0.0988 (mV). The magnetization curve aims to analyze the magnetic properties of particles. For biopurification, it is necessary to have superparamagnetic properties. This phenomenon occurs with materials that need to be under the action of a magnetic field in order to have better magnetic response. When the MPs were separated from magnetic field, the magnetization disappears. As it is observed from the graph, both types of particles present superparamagnetic properties. As such, the particles can be dissolved in solution without the presence of a magnet.

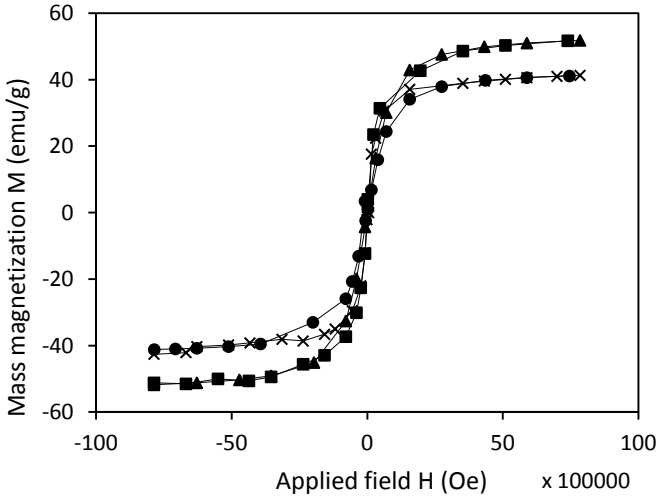


Figure 6.10 Magnetization curves for four types of particles

6.11 Antibody separation study of A2C7I1 using HGMS

The produced MPs were first tested for binding to pure solutions of human IgG (hIgG) and bovine serum albumin (BSA). The MPs samples binding capacities were shown in figure-. Final modified magnetic particles bind 20 mg of BSA per gram of MP whereas able to elute around 12 mg of BSA per gm of MP which is one of the effective and beneficial property shown by these particles as we need as less BSA binding as possible for application purpose. IgG binding was upto 130 mg per gram of MP whereas elution was too less. This elution difficulty study results were shown in next topic for optimization to obtain effective buffer for better product recovery. The final MP-A2C7I1 particles were then studied for static binding capacity using partition equilibrium experiments with pure solutions of hIgG.

The adsorption isotherm of hIgG onto MP-A2C7I1 was fitted using Scatchard plot yielding an affinity constant (Ka) of 3.3×10^4 M^{-1} and theoretical maximum capacity (Q_{max}) was 275 mg hIgG adsorbed/g MP. For the control MP-DX-Ox the determined theoretical maximum capacity was 2.6 mg hIgG adsorbed/g MP.

After preliminary studies with pure solutions of hIgG and BSA, the MP-A2C7I1 supports were tested with a CHO cell culture supernatant by direct magnetic separation method, in order to verify the applicability of the magnetic supports to capture antibodies from complex mixtures. The elution samples were analysed BCA to quantify amount of antibodies. From the results it was observed that in direct magnetic separation process using complex crude mixture also magnetic supports retain their antibody binding capability and bound 67 mg IgG per g of support where as elution was only 38 mg/g of MP.

6.12 Conclusion:

Synthesis and study of MPs coated with APBA and modified using GLYMO led particles posses promising characteristics for application in bioseparation processes using high gradient magnetic separator. At higher volume high gradient magnetic separator study for IgG purification from crude samples boronic acid coated Magnetic particles able to bound 148 mg of IgG pe gram om magnetic particles whers as MP-EPS-ligand 22/8 and MP-A2C7I1 able to bind 92 and 67 IgG in mg/g of magnetic particles. In terms of elution of MP-BA found much more efficient than others as it able to elute 112 mg IgG per gram of magnetic particles while other elute only 78 and 38 mg/g of MP. In terms of losses from high gradient magnetic separator MP-BA found less effective as at 2% concentration of feed losses were 0.6 mg per ml at 100 volt current charge and corresponding magnetic field of 1.4 Tesla, In case of MP-EPS-ligand 22/8 land MP-A2C7I1 losses were 0.4 and 0.3 mg per ml respectively. So in terms of performance in high gradient magnetic separator MP-A2C7I1 found to be effective but their IgG separation capacity was around half than that of MP-BA.

These all high gradient magnetic separation experiments were carried out by removing the stainless steel wire mesh inside the HGMS column. In presence of wire mesh separation was highly effective at too less magnetic field without loss of magnetic particles from HGMS but removal of magnetic

particles from stainless steel wire mesh inside the column becomes highly difficult and need almost equal or more quantity of water for recovery of magnetic particles which completely disturbs separation role of high gradient magnetic separator.

References:

1. An X., S. Z., Zeng H.,. (2003). Preparation of highly magnetic chitosan particles and their use for affinity purification of enzymes. Journal of Chemical Technology & Biotechnology, 78(5), 596-600.
2. Bandeira V., P. C., Rodrigues A.F., (2012). Downstream processing of lentiviral vectors: releasing bottlenecks. Hum. Gene Ther. Method, 23(4), 255-263.
3. Becker, J. S., O.R.T. Thomas, and M. Franzreb. (2009). Protein separation with magnetic adsorbents in micellar aqueous two-phase systems. Sep. Purif. Technol., 65, 46-53.
4. Bensch M., S. B., Hubbuch J., (2007). High throughput screening techniques in downstream processing: preparation, characterization and optimization of aqueous two-phase systems. Chem. Eng. Sci., 62(7), 2011-2021.
5. Bhambure R. et. al. (2011). High-throughput process development for biopharmaceutical drug substances. Trends Biotechnol., 29, 127-135.
6. Bolton G.R. et. al. (2011). Addressing the challenges in downstream processing today and tomorrow. Biopharm Int., 24, S8-S15.
7. Chen S. et. al. (2009). A screening paradigm for the design of improved polymer-coated superparamagnetic iron oxide nanoparticles. . Journal of Materials Chemistry, 19(35).
8. Chusainow J. (2009). A study of monoclonal antibody producing CHO cell lines - what makes a stable high producer? Biotechnol. Bioeng.,102, 1182-1196.
9. Dhadge V.L., H. A., Azevedo A.M., Aires-Barros MR., Roque C.A.,. (2013). Boronic acid modified magnetic particles for antibody purification. Royal society interface.
10. Dhadge V.L., P. M., Maria Reis, Azevedo A.M., Aires-Barros MR., Roque C.A.,(2014). Green polymer coated magnetic particles at the interface of biopurification. Royal society interface, Under review.
11. Dhadge V.L., A. A. M., Aires-Barros MR., Roque C.A., (2014). Magnetic aqueous two phase fishing A hybrid process technology for antibody purification. J. Chromatogr A.
12. Frakas T., S. H., Tjerneld F.,. (1996a). Partitioning of bmannanase and a-galactosidase from Aspergillus niger in Ucon/Reppal aqueous two phase system. Bioseparation, 6, 147-157.
13. Freitas S. et. al. (2009). Alternatives for the intermediate recovery of plasmid DNA: performance, economic viability and environmental impact. Biotechnol. J., 4, 265-278.
14. Goloverda G. et. al. (2009). Synthesis of ultrasmall magnetic iron oxide nanoparticles and study of their colloid and surface chemistry. . Journal of Magnetism and Magnetic Materials, 321(10), 1372-1376.
15. Heebol-Nielsen A., J. S., Hobley TJ.,Thomas ORT.,(2004). Superparamagnetic cation-exchange

adsorbents for bioproducts recovery from crude process liquors by high gradient magnetic fishing. Sep Sci Technol, 39, 2891-2914.

16. Heeboll-Nielsen A., D. M., Hubbuch JJ., Thomas ORT.,(2004). Superparamagnetic adsorbents for high gradient magnetic fishing of lectins out of legume extracts. Biotechnol Bioenng, 87, 311-323.
17. Horák D. et. al. (2007). Preparation and properties of magnetic nano- and microsized particles for biological and environmental separations. Journal of Separation Science, 30(11), 1751-1772.
18. Hubbuch J.J. and Thomas O.R.T. (2002). High gradient affinity separation of trypsin from porcine pancreatin. Biotechnol Bioeng, 79, 301-313.
19. Jonsson M. and Johansson H.O. (2003). Protein partitioning in thermoseparating systems of a charged hydrophobically modified ethylene oxide polymer. J Chromatogr A 983, 133-144.
20. Kandula S. et. al. (2009). Design of a filter train for precipitate removal in mAb downstream processing. Biotechnol. Appl. Biochem., 54, 149- 155.
21. Kuczewski M. et. al. (2011). A single-use purification process for the production of a monoclonal antibody produced in a PER.C6 human cell line. Biotechnology Journal, 6(1), 56-65.
22. Kulkarni N., V. A., Rao M.,(1999). Biochem. Biophys. Res. Commun., 225, 274-278
23. Kumar A., W. P. O., Kepka C., Galaev I.Yu, Mattiasson B., (2003). Purification of histidine-tagged single chain Fvantibody fragments by metal chelate affinity precipitation using thermo-responsive copolymers. Biotechnol Bioeng, 84, 495-503.
24. Liu HF., M. J., Winter C., (2010). Recovery and purification process development for monoclonal antibodies. mAbs, 2, 480-499.
25. Liu J. et. al. (2009). Comparison of camelid antibody ligand to Protein A for monoclonal antibody purification. Biopharm. Int. , 22, 35-43.
26. Meyer A., H. D., Gomes CSG, Hobley TJ, Thomas ORT, Franzreb M,. (2005). Demonstration of a strategy for product purification by high gradient magnetic fishing. Biotechnol Prog, 21, 244-254.
27. Muller-Spath T. et. al. (2008). Chromatographic separation of three monoclonal antibody variants using multicolumn countercurrent solvent gradient purification. Biotechnol. Bioeng., 100, 1166-1177.
28. Muller-Spath T, A. L., Strohlein G, Kornmann H, Valax P, Delegrange L, Charbaut E, Baer G, Lamproye A, Johnck M. (2010). Two step capture and purification of IgG2 using multicolumn countercurrent solvent gradient purification (MCSGP). Biotechnol Bioeng(107), 974-984.
29. Nfor B.K. et. al. (2012). Model-based high-throughput process development for chromatographic whey protein separation. Biotechnol. J. , 7, 1221-1232.
30. Nfor B.K. et. al. (2013). Model-based rational methodology for protein purification process synthesis. Chem. Eng.Sci., 89, 185-195.
31. Nishio K. et. al. (2008a). Development of novel magnetic nano-carriers for high-performance affinity purification. Colloids and Surfaces B: Biointerfaces, 64(2), 162-169.

32. Öztürk, N., et al.,2007.Silane-Modified Magnetic Beads: Application to Immunoglobulin G Separation Biotechnology Progress, 23(5), 1149-1156.
33. Poechlauer P., M. J., Broxterman R, Gregertsen B, Ridemark M. . (2012). Continuous processing in the manufacture of active pharmaceutical ingredients and finished dosage forms: an industry perspective. Org. Proc. Res. Devel., 16(10), 1586-1590.
34. Rahman M.M. et. al. (2011). Highly temperature responsive core-shell magnetic particles: Synthesis, characterization and colloidal properties. Journal of Colloid and Interface Science, 360(2), 556-564.
35. Rito-Palomares M. (2004). Practical application of aqueous two-phase partition to process development for the recovery of biological products. J. Chromatogr. B, 807, 3-11.
36. Rogers M. et. al. (2009). Development of a rapid sanitization solution for silica-based protein A affinity adsorbents. J.Chromatogr. A 1216, 4589-4596.
37. Roobol - Boza M., D. V., Doverskog M., Barrefelt A., Lindqvist F., Oppermann K., Tjerneld F.,. (2004). Membrane protein isolation by in situ solubilization, partitioning and affinity adsorption in aqueous two-phase systems. J. Chromatogr. A, 1043, 217 - 223.
38. Shukla AA. (2007). Downstream processing of monoclonal antibodies - application of platform approaches. J. Chromatogr. B, 848, 28-39.
39. Shukla A and Kandula J. (2008). Harvest and recovery of monoclonal antibodies from large-scale mammalian cell culture. Biopharm. Int., 7(34-45).
40. Tugcu N. et. al. (2008). Maximizing productivity of chromatographic steps for purification of monoclonal antibodies. . Biotechnol. Bioeng., 99, 599-613.
41.Vogel J.H. et. al. (2012). A new large-scale manufacturing platform for complex biopharmaceuticals. Biotechnol. Bioeng. , 109, 3049-3058.
42. Xu H. et. al. (2011). Antibody conjugated magnetic iron oxide nanoparticles for cancer cell separation in fresh whole blood. Biomaterials, 32(36), 9758-9765.
43. Zhao G. et. al. (2009). Ligands for mixed-mode protein chromatography: principles, characteristics and design. J. Biotechnol.(144), 3-11.

CHAPTER 7: CONCLUDING REMARKS

Thesis provides excellent insight for magnetic nanoparticle manufacture, design, development and modification for separation and purification of monoclonal antibodies which are demanding biopharmaceutical products from market point of view. Amino phenyl boronic acid coated magnetic particles modified using GLYMO exhibit promising characteristics for application in bioseparation processes. Human IgG purification will be most suitable desirable option from a separation point of view. Synthesis and modification of magnetic support using the methods described in this thesis showed encouraging results in terms of production and utilization efficiency. The support showed superior performance under elution conditions despite the absorption of some quantity of undesired proteins. Nevertheless, the overall performance for human IgG separation was better than expected. One more advantage of this support is that, it showed low nonspecific adsorption in the presence of BSA and no major loss of the capacity of the support when reused up to five times.

Hybrid and integrated processes plays important role in production of biopharmaceutical products with improved yield as well as purity at lower cost. As upstream process development work for biopharmaceuticals especially for monoclonal antibodies; enzymes and proteins achieved maximum success in terms of yield as well as productivity the downstream processing still trapped in expensive chromatographic techniques. In order to make downstream process robust and cost effective hybrid process technologies found to have revolutionary potential. As a individual unit operation aqueous two phase extraction have competitive advantages in terms of host protein removal and product recovery but in terms of purity it has some limitations which will be easily overcome by integrated hybrid technologies.

Fucose-containing extracellular polysaccharide produced by *Enterobacter* A47 (DSM 23139), shows superior suitability for utilization as a coating material for MPs. EPS support found to be promising polymer for magnetic particle coating and modification due to the simplified synthesis, low cost, high stability and reduced non-specific adsorption. The excellent capability of EPS coated MPs for the covalent attachment of a synthetic affinity ligand make these particles challenging for recovery of antibodies. EPS coated MPs also shows applicability for use in integrated process technology which joins magnetic separation process with aqueous two phase extraction for the purification of human antibodies. The ATPE process composed of 8 % PEG and 5 % Dextran afforded high recovery yield in presence of EPS-22/8 coated MPs.

For higher volume processing capabilities using high gradient magnetic separator for IgG purification from crude samples boronic acid coated Magnetic particles able to bound 148 mg of IgG per gram of magnetic particles where as MP-EPS-ligand 22/8 and MP-A2C7I1 able to bind 92 and 67 mg IgG in mg/g of magnetic particles. In terms of elution of MP-BA found much more efficient than others as it able to elute 112 mg IgG per gram of magnetic particles while other elute only 78 and 38 mg/g of MP.

In terms of losses from high gradient magnetic separator MP-BA found less effective as at 2% concentration of feed losses were 0.6 mg per ml at 100 volt current charge and corresponding magnetic field of 1.4 Tesla, In case of MP-EPS-ligand 22/8 and MP-A2C7I1 losses were 0.4 and 0.3 mg per ml respectively. So in terms of performance in high gradient magnetic separator MP-A2C7I1 found to be effective but their IgG separation capacity was around half than that of MP-BA.

Still, there is scope for improvement regarding mechanical stability, higher magnetization capability and better functionalization property. Boronic acid functionalization of magnetic particles is a promising tool to achieve higher antibody binding capability according to their use for analytical or industrial purposes. This tuning will affect and consequently improve, the efficiency of antibodies binding since less time consuming functionalisation capability of magnetic particles for ligand attachment and antibody capture. Moreover, the scale up of these magnetic supports is still limited because of requirement of more magnetic strength for separation using high gradient magnetic separator and therefore this is an area that must be addressed for optimization in order to definitely push magnetic materials applicability to industry.