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Development of immuno- nanoparticles for targeting schistosoma parasites

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Development of Immuno- Nanoparticles for Targeting *Schistosoma* Parasites

A Thesis Submitted to

The Biotechnology Graduate Program

In partial fulfillment of the requirements for

The degree of Master of Science in Biotechnology

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Abstract

Praziquantel (PZQ) is the drug of choice for treatment of schistosomiasis since its discovery in 1972.[1,2] According to World Health Organization (WHO), schistosomiasis is the second most prevalent parasitic disease worldwide that has no effective vaccine yet. Schistosoma parasites infect more than 200 million people in about 76 countries and about 700 million people are at risk. [3,4] PZQ is not only used for treatment of schistosomiasis, but it is also used for control of other parasitic diseases and it is included on the Model List of Essential Medicines of WHO.[5,6]

Parasitic diseases, despite their significant global burden, have been rather under researched by drug discovery companies as compared to other diseases mainly due to complexities of parasitic infections and lack of economic motivations. In such scenario, nanomedicine has a crucial role to play in improvement of the current anti-parasitic agents by their delivering in a targeted manner to the schistosome parasites in host blood circulation. Hence, selective drug delivery is an important approach with great potential for overcoming problems associated with the systemic toxicity and poor bioavailability of PZQ.

This study aims at overcoming the inherent drawbacks of PZQ through formulation, optimization and evaluation of PZQ-loaded biodegradable PLGA "Poly (D,L lactide-co-glycolide)" nanoparticles (NPs) that were surface conjugated with polyclonal antibodies (pAb) specific for schistosome surface antigens (sAg) for active targeting and treatment of schistosomiasis.

PZQ was encapsulated in biodegradable PLGA NPs using single emulsion-solvent evaporation method. Various parameters were investigated and optimized like drug: polymer ratio, surfactant concentration and osmotic effect of adding salts to the external aqueous phase. PZQ-loaded NPs were characterized for drug content and drug release rate using HPLC, particle

size, particle size distribution and ζ-potential using dynamic light scattering, surface morphology using scanning electron microscopy (SEM) and atomic force microscopy (AFM), and drug physical and chemical integrity using differential scanning calorimetry (DSC) and Fourier transform infrared (FTIR) spectroscopy. Spherical PLGA NPs with smooth and discrete surface were prepared. The physicochemical properties of the prepared NPs were investigated and the optimum formulation was selected. The selected formulation was monodispersed NPs 200.8±4.02nm in diameter and negatively charged surface (-21.03±3.08mV). PZQ- loaded PLGA NPs showed sustained release profile from the optimum formulation with the highest encapsulation efficiency and drug loading (94.19±3.46% and 47.09±3.46% respectively).

Specific anti- schistosomal pAb specific against schistosome sAg was prepared, purified and characterized. Surface functionalization of the optimized PZQ-loaded PLGA NPs with anti-schistosomal pAb was then achieved using both physical adsorption and chemical conjugation methods using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) mediated cross linking agents. The pAb- conjugated immuno-NPs showed higher coupling efficiency than the pAb- adsorbed NPs (89.31±1.78 and 64.2±2.87%, respectively). Re-evaluation of the physical properties of NPs and the reactivity of pAb after coupling reactions was performed. The immuno-NPs indicated optimum properties with preserved bioactivity of the attached antibodies.

Thus, it was concluded that pAb-conjugated PLGA NPs provide an efficient and targeted delivery of PZQ, presenting a potential preliminary delivery system for treatment of Schistosomiasis in near future.

Key Words

Praziquantel – Schistosomiasis – PLGA – Nanoparticles – Immuno-nanoparticles –Adsorption – EDC/ NHS – Surface Functionalization –Schistosomicidal

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List of Abbreviations

- AFM — Atomic Force Microscopy
- ANOVA — Analysis of Variance
- AUC — Area Under the Curve
- BSA — Bovine Serum Albumin
- CFA — Complete Freund's Adjuvant
- CP — Cysteine Proteinases
- DCM — Dichloromethane
- DEAE — diethyl amino-ethyl
- DL — Drug Loading
- DLS — Dynamic Light Scattering
- DSC — Differential Scanning Calorimetry
- EA — Ethyl Acetate
- EDC — 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide Hydrochloride
- EE — Encapsulation Efficiency
- ESD — Emulsion- Solvent Diffusion
- ESE — Emulsion- Solvent Evaporation
- E/S — Excretory-Secretory
- FBS — Fetal Bovine Serum
- FTIR — Fourier Transform Infrared
- GC — Gas Chromatography
- HPLC — High Performance Liquid Chromatography
- HEPES — N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
- HRP — Horse Raddish Peroxidase
- IFA — Incomplete Freund's Adjuvant
- IgG — Immunoglobulin G
- LDA — Laser Doppler Anemometry
- M.Wt. — Molecular Weight
- NHS — N-Hydroxysuccinimide
- NPs — Nanoparticles
- NTD — Neglected Tropical Disease
- OD — Optical Density
- OFAT — One Factor At Time
- OPD — Ortho-Phenylenediamine Dihydrochloride
- pAb — Polyclonal Antibodies
- PCS — Photon Correlation Spectroscopy
- PDI — Polydispersity Index
- PLGA — Poly (D,L Lactide-Co-Glycolide

- PVA Polyvinyl Alcohol
- PZQ Praziquantel
- RPMI Roswell Park Memorial Institute
- SAA Surface Active Agent
- sAg Surface Antigens
- SDS Sodium Dodecyl Sulfate
- SDS-PAGE Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
- SEM Scanning Electron Microscopy
- SBSC Schistosome Biological Supply Center
- TBRI Theodore Bilharz Research Institute
- WHO World Health Organization

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Introduction

Schistosomiasis (Bilharziasis) is a blood disease caused by a trematodal parasite of genus *Schistosoma* (Bilharzia). Bilharzia was named after the German surgeon Theodore Bilharz who discovered it in 1851 in Egypt and found that *Schistosoma haematobium* is the causative agent of the disease. Schistosomiasis is considered to be the second most destructive disease, after malaria, in the tropical countries. [7–9] It is an epidemic disease that affects more than 200 million people worldwide and more than 700 million are at risk in more than 76 different countries. Mortality has been estimated to be 280,000 deaths/ year in Sub-Saharan Africa. Schistosomiasis presents in wide spectrum disease including both acute and chronic forms, affecting multiple target organs. [4,10,11] **Figure 1** shows the worldwide epidemiological distribution of schistosomiasis.

There are more than five different species that cause schistosomiasis. The most widely spread species is *Schistosoma mansoni* which is found in Africa (some subtropical and tropical regions), the Caribbean, the Middle East and South America. The second species is named *S. haematobium* and is spread in Egypt, Sub-Saharan Africa, India North Africa and Middle East. The third type is called *S. japonicum* which is only prevalent in Asia. The fourth type, *S. intercalatum,* is present more in West and Central Africa, while the fifth type is called *S. mekongi* and is only present in Cambodia and Laos. [3,12,13]

The life cycle of all species of *Schistosoma* is the same. *Schistosoma* worms are either males or females. The female worm is thin and tall and stays in a groove in the male body called the gynecophoric channel. *Schistosoma* worms have two hosts; the primary host (human) and the secondary host (fresh water snails). Sexual life cycle takes place in humans while asexual one takes place in the snails. Not all snails act as a secondary host, only some as *Biomphalaria* for *S. mansoni*, *Bulinus* for *S.haematobium* and *Oncomelania* for *S. japonicum*.[3,12–15] **Figure 2** is an illustration of *Schistosoma* life cycle.

As a neglected tropical disease (NTD) and like many other “damaging” parasitic diseases, no enough efforts go for finding solutions for suffering of such a huge number of people worldwide.[16] Since its detection on 1851, no treatment was efficient enough for the

control of such an infection until the discovery and production of praziquantel (PZQ) in 1972 by "Bayer and Merck" pharmaceutical company.

Praziquantel is a pyrazino-isoquinolone derivative (**Figure 1.15**) characterized by its broad spectrum activity against a wide range of parasites other than *Schistosoma*. The "polyparasitism" capacity of PZQ has made it the drug of choice for treatment of many parasitic infections. [17–19]

PZQ greatly helped in controlling the spread of schistosomiasis in many countries. However, it did not prevent its spread because of several interconnected factors. One reason is the development of water resources in several tropical countries which has probably contributed to maintain these figures at relatively constant – if not increasing – levels in recent years. As these water resources contribute to the spread of the not- easy to control snails that act as the intermediate hosts.[5,17,17]

Since no vaccine exists against schistosomiasis, chemotherapy is the main approach for schistosomiasis control till nowadays. PZQ is currently the only available anti-schistosomal drug and it is distributed mainly through mass administration programs to millions of people every year. By time and by depending on only one drug for treatment and control of such an infectious disease (Massive and repeated use on large numbers of individuals), the development of drug resistance is a much feared possibility. Resistance of schistosome parasites against PZQ started already to appear in different places in the world.[20,21]

Development of resistance against PZQ is a serious challenge that alarms the scientific community to find a solution and fasten the research done to discover new chemotherapeutic agents or vaccine for control of schistosomiasis. The issue is that the process of discovery of novel drug and its approval by FDA is a very long procedure that needs several years and billions of dollars. To that time, there should be other solutions.

However, development of resistance is not the only problem associated with PZQ. There are many other drawbacks linked with this drug. For example, the main available dosage form (DF) of PZQ in the market is the oral tablets. PZQ suffers from first pass metabolism by the liver when taken orally that converts about 80% of the administered dose to completely inactive metabolites. This necessitates its administration in very high doses to compensate for the

metabolized and inactivated part of it; which, in turn, exposes the patient to its side effects, helps the parasite to develop resistance against the drug, in addition to its socio-economic impact. As it will be more costing and will not be available for all who need it especially that this disease is epidemic in very poor countries as mentioned above. [22–24]

Other problems associated with PZQ being administered in a traditional oral dosage form is it distribution in the whole body exposing different organs to the drug while not in need for it. Its fast elimination necessitates re-administration as patients are susceptible for exposure and re-infection with the parasite. Another major flaw of PZQ is its lack of efficacy against the immature stages of the parasite.

All these reasons push scientists for finding a solution for such a problem. Since finding a new drug from scratch will let people suffer for long time waiting for it, other solutions should be found all that time. Various options may be useful.

Firstly, development of an effective vaccine would be great; unfortunately, no successive trial was achieved. This might be due to the complexity of the host – parasite interaction as Schistosoma worms can evade the host immune response and live in the host blood circulation for 5 to 20 years. The host defense mechanisms start only when eggs were being laid or against the dead worm after treatment.[22–28]

Another possible approach is finding new indications (schistosomicidal effect) of already FDA approved drugs for treatment of other diseases. A large number of compounds have been tested as potential anti-schistosomal agents. For example, many antimalarial drugs such as mefloquine and artemether showed anti-schistosomal effects *in vitro* and on infected mice. Some of them are promising, but none so far represents a suitable substitute or adjunct to PZQ. [29–33] Research should be continued to select the most effective drug that we could depend on it for treatment of schistosomiasis either alone or as combination therapy with PZQ. The research of new anti-schistosomal compounds is an imperative and urgent matter.[17]

More realistic and hopeful solution is overcoming the inherent problems and improving the physico-chemical properties of the currently used and most effective drug, PZQ. This could be achieved by applying and benefiting from the recent advances in bio- nano technology solutions to design and develop targeted nano-carrier drug delivery systems that will solve many

of the inherent drug drawbacks. Then, further formulate them in suitable dosage forms to be available for suffering patients and people worldwide.

Nanotechnology is involved in the design, synthesis, characterization and application of materials and devices whose smallest functional organization is on the nanometer scale at least on one dimension. The clinical applications of nanotechnology in the pharmaceutical field either take advantage of the unique properties of drugs in the nano-range or the incorporation of drugs into nano-carriers to control drug release, drug targeting, or salvage of drugs with low bioavailability. Nanocarriers can be made from a variety of materials such as lipids (liposomes, solid lipid nanoparticles), polymers (nanoparticles, micelles, dendrimers) and inorganic carriers (gold nanoparticles, carbon spheres and nanotubes). According to the manufacturing methods and materials used, these carriers may have different shapes and sizes with distinct properties.[34–38]

Nanoparticles (NPs) in the field of nanotechnology have been emerging as key mechanisms by providing a targeted approach for efficient delivery of conventional chemotherapeutic drugs for cancer therapy. This targeted approach leads to the delivery of drugs, in low dosages, in a sustained manner for an efficient action within the body. NPs are nano-sized stable, colloidal or solid particles, generally made of biodegradable polymer or lipids. They entrap or encapsulate the active principal or adsorb or attach to their surface. [38–41]

In our case, to overcome the inherent drawbacks of PZQ so that it could be more effective chemotherapy for control and treatment of schistosomiasis, PZQ could be first loaded into polymeric nanoparticulate carriers.

Polymeric NPs are considered a versatile medium for the delivery and monitoring of therapeutic compounds in vivo due to their enhanced biological stability and extended in vivo circulation. They account for more than 80% of the available therapeutics in clinical use. The increased interest in using biodegradable nanoparticles for drug delivery arises from their three main basic properties. First, their small particle size facilitates their penetration into smaller capillaries and their subsequent uptake by the cells, ensuring efficient drug accumulation at the target sites. Secondly, their biodegradation allows sustained drug release within the target site

over a period of days or even weeks. Thirdly, the possibility of their surface functionalization and modification allows active drug targeting via attachment of specific ligands.[34–38,42–44]

Both synthetic and natural polymers have an important role as biomedical materials for drug delivery, yet synthetic biodegradable polymers such as poly(amides), poly(amino acids), poly (alkyl-a-cyano acrylates), poly(esters), poly (orthoesters), poly(urethanes) and poly(acrylamides) have been increasingly used to prepare various drug-loaded devices. These polymers are free from most of the problems commonly associated with natural polymers (albumin, chitosan, etc.) such as immunogenicity, variability in purity across groups, complex structure, strength inadequacy and difficulty in controlling material degradability. Among the synthetic polymers, the thermoplastic aliphatic poly(esters) such as polylactic acid (PLA), polyglycolic acid (PGA) and, especially PLGA, have generated tremendous interest due to their excellent biocompatibility, biodegradability and toxicologically safe by-products.[45–47]

PLGA is a copolymer of lactic acid and glycolic acid. Depending on the used ratio of lactide to glycolide, different forms of PLGA can be obtained. They are usually identified with regard to the monomers ratio used (e.g., PLGA 75:25 identifies a copolymer whose composition is 75% lactic acid and 25% glycolic acid). PLGA is one of the most successfully used biodegradable polymers for the development of nano-medicines. It undergoes hydrolysis in the body to produce the biodegradable metabolite monomers, lactic acid and glycolic acid (**Figure 1.17**) which are endogenous and easily metabolized by the body via kreb's cycle.[40,41]

The specificity of delivery using nanoparticles was initially a coincidental property, however active targeting has now become a vital concept in therapeutic research. **Active targeting** of nano- particles to particular sites in the body can be achieved using target-specific ligands or antibodies that can be attached onto the nanoparticulate surface. These modifications direct the drug to the specific cell for the desirable action in low doses.[48]

As a result, surface functionaliztion of the PZQ-loaded PLGA NPs with a specific ligands against Schistosoma parasites may produce a more efficient form of treatment delivering the active drug to the parasites in the host and exerting the anti- schistosomal actions over a sustained period of time without causing undesired side effects.

We aim at developing nano-carriers (PLGA NPs) loaded with smaller doses of the drug (PZQ) and surface- coated with specific antibodies (Anti- *Schistosoma* polyclonal antibodies) for active targeting of schistosome surface antigens.

The work in this thesis was divided into two chapters and three main studies:

1. ***Chapter One:*** Development and Optimization of PZQ-loaded PLGA Nanoparticles *(Study One)*
2. ***Chapter Two:*** Development of Immuno—Nanoparticles for Active Targeting of *Schistosoma* Parasites
   A. Preparation of Anti-schistosomal Polyclonal Antibodies against *Schistosoma* Surface Tegumental Antigens *(Study Two)*
   B. Preparation and Characterization of PLGA Nanoparticles Surface – Modified With Purified Anti- *Schistosoma* Polyclonal Antibodies *(Study Three)*

Chapter One: Development and Optimization of PZQ-loaded PLGA Nanoparticles

Section I: Methodology[*]

[*] **All materials and equipment used in this study were described in *Appendix I* and *Appendix II* respectively.**

In this section, PLGA NPs either plain or PZQ-loaded were prepared. The effect of various formulation and experimental variables on different NPs properties like size, charge, drug encapsulation efficiency and morphological characteristics were investigated. The levels of tested variables have been optimized to obtain NPs having the highest possible loading of PZQ with optimum size and release profile.

I.A. Preparation of Free and PZQ-loaded PLGA Nanoparticles

PLGA NPs were prepared and PZQ was encapsulated into the NPs. As several factors affect the characteristics of the prepared NPs as shown in **Figure 1.1**, the work was divided into five main studies as shown in **Figure 1.2**.

I.A.1. Study I: Preliminary Study

The aim of this study was first to select the method of preparation of PZQ- loaded PLGA NPs. Then, fabrication parameters and experimental conditions affecting the chosen method will be optimized to be fixed and used throughout all the successive studies for NPs preparation. Optimization of various parameters was done with respect to particle size and size distribution, charge, drug entrapment efficiency and particle morphology.

Accordingly, the design of this study follows "One Factor at Time (OFAT)" stepwise approach. In which, a single factor is under study in each experiment while, other factors have fixed levels throughout all experiments. The chosen level of each investigated factor is used and fixed throughout all successive experiments. **Table 1.1** summarizes the investigated factors in this study and the composition of the prepared formulae.

There are several methods for preparation of PLGA NPs.[49,50] As shown in **Table 1.1**, the first experiment **(Expt. I-1)** was to select one of three of the most commonly used methods of preparation of PLGA NPs to be further used throughout this work. Typically, the three methods are based on preparation of an organic phase consisting of polymer (PLGA) dissolved in organic solvent containing, or not, the drug. This organic phase is added to an aqueous phase containing a surfactant to form an emulsion. This emulsion is broken down into nano-droplets by applying external energy and these nano-droplets form NPs upon evaporation of the highly volatile organic solvent. The solvent is evaporated leaving behind a colloidal suspension of PLGA NPs in water. [41,49,51]

Although the three methods have almost the same principle in NPs formation, they are different mainly because of the different type of organic solvent used and the technique (External Energy) applied for emulsion formation.[49,50] To elucidate, if the organic solvent is completely immiscible with the aqueous phase like dichloromethane (DCM), the method is called **emulsion-solvent evaporation (ESE)**. While, if it is partially miscible like ethyl acetate (EA), it is known as **emulsion- solvent diffusion (ESD)** method. When the solvent is miscible with the aqueous phase, it is named as **nanoprecipitation or solvent displacement technique**.[49,52,53] **Figure 1.3** is a schematic representation of the preparation methods.[53–55]

I.A.1.1. Emulsion – Solvent Evaporation (ESE) Method

Using single oil in water (o/w) emulsion – solvent evaporation (ESE) technique, formula **P1** was prepared. [53,56–58]Briefly, PZQ- loaded PLGA NPs were formed by dropwise addition of an organic phase containing completely dissolved 50mg PZQ and 300mg PLGA in 6ml DCM to an aqueous phase solution containing 1% w/v PVA while homogenization at 20000 rpm for 20 minutes over an ice bath. Upon emulsification, DCM was evaporated under reduced pressure using rotavap (45^{o}C, 14mm Hg) for 20 minutes. The formed NPs were collected by centrifugation at 20000 rpm for 20 minutes at 4^{o}C and washed twice. Then, NPs were re-suspended in deionized water for further characterization.

I.A.1.2. Emulsion – Solvent Diffusion (ESD) Method

PZQ- loaded PLGA NPs (Formula **P2**) were prepared by emulsification solvent diffusion (ESD) method. [50,59,60]Briefly, the organic phase containing 50mg PZQ and 300mg PLGA in ethyl

acetate (EA) was injected slowly to an aqueous solution of 1% w/v PVA while mixing using homogenizer at ascending speed order from 5000, 10000 and 20000 rpm for 3 minutes each. An excess amount of water equal to the original aqueous phase (114ml) was added slowly while homogenization at 20000 rpm for 3 minutes. The solvent was evaporated under reduced pressure using rotavap (60^{o}C, 21mm Hg). Nanoparticles were purified by 20 min centrifugation at 20000 rpm at 4^{o}C and washed twice then, re-suspended in water for further characterization.

I.A.1.3. Nanoprecipitation Method

PZQ- loaded PLGA NPs (Formula **P3**) were prepared by nanoprecipitation technique. [61,62]Briefly, a 6 ml organic phase (acetone) of PZQ and PLGA was slowly dropped with a constant speed (0.5 mL/min) to a 114 ml aqueous solution of 1% w/v PVA with continuous magnetic stirring at 800 rpm. The solvent (acetone) was evaporated at room temperature (25°C) for 24 hours under magnetic stirring. Nanoparticles were purified by 20 min centrifugation at 20000 rpm then washed twice and re-suspended in water for further analysis.

Formulae **P4** to **P21** were prepared by ESE method as mentioned in **Table 1.1**. The aim of Experiments **I-2** to **I-8** is to study the effect of various processing parameters and experimental conditions of ESE method on the properties of NPs e.g. mean size, size distribution, charge, drug content, drug loading and surface morphology.

I.A.2. Study II: Screening Phase

This phase is conducted to study the effect of different material characteristics on the size, size distribution, charge and morphology of the prepared NPs. The experimental conditions and other formulation parameters selected from the preliminary phase were used in the preparation of all coming formulae. **Table 1.2** summarizes the studied variables in this phase and the composition of formulae **S1-S10**. The formulations were evaluated for particle size, size distribution and charge.

Typically, modified single oil in water (o/w) emulsion – solvent evaporation (ESE) technique was used for the preparation of PLGA nanoparticles. Briefly, a specified volume of the organic solvent dichloromethane (DCM) containing completely dissolved PLGA was slowly poured into aqueous poly vinyl alcohol (PVA) solution and leucine (0.01%, w/v) while mixing in homogenizer at ascending speed order from 5000, 10000 and 20000 rpm for 3 minutes each.

The resulting emulsion was sonicated using a probe sonicator. During homogenization and sonication, the whole system was placed in an ice bath to prevent any loss of the oil phase due to evaporation. The organic phase was evaporated on a magnetic stirrer under atmospheric conditions till complete evaporation of DCM accompanied by the formation of colloidal suspension of PLGA nanoparticles (NPs). The nanoparticles were recovered by 30 min centrifugation at 11,000 rpm then washed twice in order to remove the adsorbed PZQ and any excess surfactant from the surface of NPs. The washing solutions were eliminated by a further centrifugation as described previously. The purified nanoparticles were either freshly used for further investigation or re-suspended in water containing 0.2% w/v sucrose (lyoprotectant) and lyophilized using a freeze-drying system yielding powdered nanoparticles which were stored in desiccator to be used for further analysis. All nanoparticle suspensions were prepared in triplicate.

I.A.3. Study III: Validation Phase I

This study was conducted to validate the selected variables from both the preliminary and screening phases. The validation was performed using both PLGA 50:50 and PLGA 75:25 as shown in **Table 1.3** to see the their effect on drug encapsulation and drug loading.

PZQ- loaded PLGA NPs were prepared by the same method mentioned in section **I.A.2**. However, PZQ was dissolved first via vortexing in the specified volume of the organic solvent dichloromethane (DCM), containing PLGA. A non-medicated preparation (Blank) was prepared to study the effect of drug on nanoparticles character and find the interference from nanoparticles components at drug wavelength.

I.A.4. Study IV: Optimization Phase

This phase was required to be conducted to optimize the drug encapsulation efficiency and drug loading. Various parameters that might affect the PZQ encapsulation into PLGA NPs were studied using OFAT approach. All the experiments are conducted by varying one of the parameters while keeping all the other parameters at a set of standard conditions as shown in **Table 1.4**. NPs were prepared as mentioned previously and illustrated in **Figure 1.4**.[58,63] The formulations were optimized on the basis of size, size distribution, charge, entrapment efficiency and drug loading.

I.A.5. Study V: Validation Phase II

All selected factors combined were validated to reach the optimized formulae with the highest PZQ encapsulation efficiency and loading into PLGA NPs with optimum size and size distribution. Also, the effect of initial drug weight on NPs properties was studied as shown in **Table 1.5**.

I.B. Characterization of Blank and PZQ- Loaded PLGA Nanoparticles

The method used for preparation of NPs was optimized with respect to various fabrication parameters and experimental conditions over five successive studies as described earlier. The prepared NPs were characterized on the basis of particle size, size distribution, charge, drug entrapment efficiency and drug loading till the formula with the highest possible drug encapsulation efficiency and drug loading with optimum size and homogenous size distribution was obtained. In addition to that, the optimized formula was further evaluated to study the PZQ release behavior from PLGA NPs, the NPs morphology using scanning electron microscopy (SEM) and atomic force microscopy (AFM), and the physical compatibility between NPs components with confirmation of PZQ encapsulation into PLGA NPs using differential scanning calorimetry (DSC) and Fourier-Transform infrared spectroscopy (FTIR).

I.B.1. Particle Size Determination and Particle Size Distribution

The hydrodynamic diameters (Z-Average) of NPs were determined by photon correlation spectroscopy (PCS) based on quasi-elastic or dynamic light scattering (DLS) using a particle size analyzer (Zetasizer). This technique yields the particle mean particle diameter and the polydispersity index (PDI); which is a dimensionless measure for the broadness of the particle size distribution. [64–66] Briefly, the freshly prepared and purified suspension of NPs was a 1/100 (v/v) diluted in MilliQ water. However, for lyophilized NPs, 1mg NPs were suspended in 5ml deionized water and shaken to ensure uniform distribution. Size measurements were performed in disposable cuvettes at a scattering angle of 90^{o} and at a temperature of 25^{o}C. For statistical analysis all samples were measured in triplicate and the average values and standard deviation of the measurements were calculated.

I.B.2. Zeta potential (ζ) Measurements

Surface Charge (ζ potential) of NPs was analyzed by laser Doppler anemometry (LDA) using the same instrument (Zetasizer) and the protocol mentioned above (section **I.B.1**). The NPs suspension was 100-fold diluted in deionized water and measured in a capillary cell (detector angle 90°, at 25°C, wavelength 633 nm). Measurements were carried out in triplicate. Results are expressed as mean ±SD.[56,67,68]

I.B.3. Quantitative determination of PZQ, Drug Encapsulation Efficiency and Drug Loading

I.B.3.1. Quantitative Analysis of PZQ

Several procedures have been developed for the quantitative determination of PZQ. These procedures include high performance liquid chromatography (HPLC), gas chromatography (GC), spectrophotometry and colorimetry. [56,69] In this study, quantitative HPLC of PZQ was performed using Agilent 1260 Infinity Quaternary LC with a Diode Array Detector DAD (set at 262 nm) and Agilent Standard Autosampler with a 100 µL loop. Chromatographic separation was accomplished using a reversed phase Agilant Zorbax SB-C18 column (150 mm x 4.6 mm id., 5 µm particle size). The flow rate was isocratic at 1 mL/min. The mobile phase was prepared by mixing acetonitrile with water 60:40 (% v/v).The UV detector was set at 262 nm. The HPLC system was operated at (30 ± 1 °C). The mobile phase was filtered through a membrane filter (0.45 µm X 47 mm) and degassed through Agilent 1260 infinity quaternary pump. The analysis required less than 3.2 min.

Stock solution of PZQ standard (500µg/mL) in acetonitrile: water (60:40) was freshly prepared accurately weighing 50 mg PZQ reference substance, transferring to a 100 mL volumetric flask followed by addition of mobile phase to make up the volume. The working solutions were prepared by diluting the stock solution in the mobile phase to prepare a set of calibration solutions of concentration ranging between 2 and 15µg/ml. A calibration curve was constructed by plotting the area under the curve (AUC) and the concentration of the drug in µg/mL. The linear regression equation was calculated. All determinations were conducted in triplicate.

The accuracy, precision of the assay and linearity of the calibration curve were determined for intra- and inter-day on three different days. The precision was expressed as the percent coefficient of variation (CV %) of each curve.

I.B.3.2. Drug Entrapment Efficiency and Drug Loading

The amount of PZQ entrapped in the nanoparticles was determined by HPLC in triplicate under the same conditions as described above (Section **I.B.3.1**). [56]The method adopted for estimating the entrapment efficiency was a direct method via drug extraction from NPs. [70] Definite amount of the precipitated NPs (1mg) were added to excess volume of methanol (10 mL). The solvent containing the NPs was vigorously stirred over shaker incubator at 300rpm for 48 hours at RT in order to completely extract out the drug. After eventual dilutions in the mobile phase, the concentration of the extracted drug measured assayed using the above HPLC method (UV detection set at 262 nm, a reversed phase C18 column (150 mm x 4.6 mm id., 5µm particle size) was used, the mobile phase consisted of a mixture of acetonitrile: water (3:2 v/v) and the flow rate was set at l mL/min) against a blank formula. The solutions were previously filtered using a membrane filter (pore size 0.45 µm X 47 mm). All measurements were done in triplicate.

The encapsulation efficiency EE (%) was calculated according to the following equation:

EE% = (Actual Drug Weight in NPs / Initial Drug Weight) X 100

The drug loading (DL) was calculated according to the following equation:

DL% = (Actual Drug Weight in NPs / Initial Drug and Polymer Weights) X 100

Also, indirect estimation of PZQ concentration in supernatant after NPs purification (Indirect Method) was done to confirm the complete extraction of the drug from NPs. In this case, the encapsulation efficiency was calculated as follows:

EE% = [(Initial Drug Weight ― Measured Drug Weight in NPs) / Initial Drug Weight] X 100

I.B.4. *In Vitro* Release Study

The *in vitro* drug release profile of entrapped PZQ from PLGA NPs was studied by dialysis bag diffusion technique. [71,72]Each dialysis bag (Cellulose membrane molecular weight cut-off: 12 kDa) was loaded with an accurately weighed amount of lyophilized NPs of optimized

preparation **(VII4)** equivalent to 500µg of drug. The particles were suspended in 2 mL of PBS (pH = 7.4). The dialysis bag was then immersed in 20 ml of release medium PBS (pH = 7.4) containing 0.02 % sodium azide as preservative at 37±1 °C and was continuously stirred on a shaker incubator at 100 rpm/min. At predetermined time intervals, aliquots of 500µL were withdrawn and replaced with the same volume of fresh dialyzing medium and the withdrawn samples were assayed for drug content against the blank using HPLC. The obtained values were corrected for the dilution used during the sampling. Experiment was performed in triplicates. Drug release data were expressed as the percentage of the cumulative amount of drug release.

I.B.5. Thermal Analysis Using Differential Scanning Calorimetry "DSC"

The physical state of the PZQ encapsulated in PLGA NPs was characterized using a differential scanning calorimetric (DSC) thermogram analysis (Perkins Elmer Differential Scanning Calorimeter). Samples of drug, polymer, SAA, physical mixture of drug and polymer, physical mixture of drug and SAA and freeze dried drug-loaded NPs were tested. Each sample (10mg) was sealed separately in a standard aluminum pan, the samples were purged in DSC with pure dry nitrogen set at a flow rate of 20 ml/ min, the temperature speed was set at 10^{o}C/min, and the heat flow was recorded from 30 to 350^{o}C. Indium was used as the standard reference material to calibrate the temperature and energy scales of the DSC instrument.[65,68,73]

I.B.6. Spectral Analysis Using Fourier-Transform Infrared Spectroscopy "FTIR"

The Fourier transform infrared (FTIR) spectra for PZQ, PLGA, PVA, physical mixture of drug and polymer, physical mixture of drug and SAA and freeze dried drug-loaded NPs were obtained using FTIR spectrophotometer for characterizing the chemical integrity of PZQ inside PLGA NPs. Briefly, the samples (3mg each) were pressed with potassium bromide (KBr) to make a pellet by applying a pressure of 300 kg/cm^{2} before obtaining their IR absorption spectra. The spectra were detected in KBr discs over a range of 4000-400 cm^{-1}. The detector was purged carefully by clean dry helium gas to increase the signal level and reduce moisture.[68,74]

I.B.7. Particle Morphology

The surface morphology of the prepared NPs was examined as follows:

I.B.7.1. Optical Microscope

For microscopic analysis, a sample of the prepared NPs dispersion was placed on a microscope slide, diluted with deionized water, examined using Leica optical microscope at a magnification of 40X and photographed for morphological evaluation.

I.B.7.2. Scanning Electron Microscopy

The surface morphology of nanoparticles was characterized by scanning electron microscopy SEM. Briefly, the NPs samples for SEM analysis were prepared either by drying a drop of nanoparticle freshly prepared suspension on a metal stub as non-aggregated particles or by affixing the NPs lyophilized powder to stubs using double-sided carbon tape. Then, the NPs were sputtered with a thin layer of gold using a current of 40 mA for 25 seconds under an argon atmosphere in a high-vacuum evaporator to make them conductive electron microscopic scanning. Observation was performed at an accelerating voltage of 10–30 kV. [66,73]

I.B.7.3. Atomic Force Microscopy

The NPs samples were examined for their shape and morphology and their surface properties were evaluated using atomic force microscope (AFM) "Vecco Atomic Force Microscope". The samples were prepared separately on sample holders (Silicon Wafers). On each holder, one drop of sample suspension was dried as non-aggregated particles and examined.[75,76]

I.C. Statistical Analysis

All experiments were carried out three times, independently. Results are expressed as mean ± standard deviation (SD). Significant statistical differences between groups were examined by applying Student's t-test for two groups' comparison and one-way analysis of variance (ANOVA) for three or more groups. The statistical significance level (*P value*) was set at ≤0.05. Statistical analysis was done to all data obtained for all NPs formulae. All tests were calculated using the software ***GraphPad Prism Software Version 6, San Diego, CA***.

Section II: Results and Discussion

In this section, PLGA NPs were prepared either un-loaded or PZQ- loaded using three different methods at the beginning: ESE method, ESD technique and nanoprecipitation method (**Figure 1.3**). Single (O/W) ESE method was selected (**Figure 1.4**).[58,63] The effect of several formulation parameters and experimental conditions affecting this technique was investigated. Their levels were selected and optimized till achieving the optimum formula with the optimized size (200.8 ± 4.02 nm) with homogenous size distribution (PDI = 0.13 ± 0.08) and the highest encapsulation efficiency and drug loading (EE% = 94.19% ± 3.47, DL% = 47.095% ± 3.47). The optimized formula was further characterized for their shape and surface morphology using SEM and AFM. They were also characterized for their physicochemical properties using DSC and FTIR. In addition, PZQ release behavior from PLGA NPs was assessed using the dialysis bag diffusion technique.

II.A. Quantitative Analysis of Praziquantel

Before preparation of PZQ- loaded PLGA NPs and to be able for their characterization, a method for quantitation of PZQ should be established and a calibration curve should be constructed to quantify the entrapped PZQ in PLGA matrix, calculate the drug loading and assess the cumulative PZQ release percent from NPs.

As mentioned in the methodology chapter, a HPLC method for determination of PZQ was used as described in the United States Pharmacopeia (USP XXVI) using as mobile phase a mixture of acetonitrile and water in the proportion of 60:40. It is worth mentioning that although the UV detector was set at 210 nm in the protocol mentioned in the US pharmacopeia, the UV detector was set at 262 nm in this method. This is because the pharmacopeia method was established for PZQ determination in tablets. [77,78]However, in the development of new dosage forms for drugs, special care must be taken in the selection of the adequate wavelength because other substances present in the formulation used as adjuvants can be absorbed in the same wavelength of the drug, interfering this in the results of the analysis. In the case of PZQ, the wavelength of 210 nm shows a high absorptivity, but in some cases it is not selective for its quantification because it depends on the excipients being used in the formulation. Some polymers, mainly polyesters, are absorbed in a wavelength of 210 nm, and as these substances are very much used in solid dosage forms, other wavelength must be selected. As validated in

many previous studies, a HPLC method for determination of PZQ which allow a better selectivity in the presence of polyesters, using a UV detector set a 262 nm.[56,68,78]

In this study, linearity of the standard calibration curve was investigated **(Figure 1.5)** and the ideal concentration range to work with was selected **(Table 1.6)**. The interval showed the desired linearity. The analytical curve for PZQ in a concentration range from 2 to15 µg/mL was linear presenting correlation coefficient (R) of 0.999 and R^2 of 0.999. The linear regression equation was found to be:

$$\mathbf{y = 6.3111\ x + 0.2549}$$

Where **y** is the area under the curve (AUC) and **x** is the concentration of standard in µg/mL.

The accuracy and precision of the assay was also determined. The intra-day assay coefficients of variation (CV %) for PZQ at 12 µg/mL (n=10) in the first day was found to be 1.86%. In the second day, it was 1.28% and in the third day, it was 2.16%. The inter-day assay was assessed on 3 individual days, the CV % was 1.77% at 12 µg/mL. These data show a remarkable degree of precision and reproducibility of the method, both within runs and between-runs.

The retention time repeatability during the precision studies was found to be good to all solutions. The retention times (t_R) of the PZQ reference substance was 3.2 min as shown in the chromatogram of PZQ reference standard **(Figure 1.6)**. The limit of detection of PZQ was approximately 1µg/mL. The limit of quantification was set at 2µg/mL being the lowest concentration used in the constructions of the standard curve. Thus, this HPLC assay was considerably simple, precise and sensitive for PZQ quantification especially that PLGA polymer did not interfere with the reading of PZQ at the selected wavelength (262nm). PlGA was separated chromatographically at earlier retention time (1.2 minutes) as shown in **Figure 1.6b**.

II.B. Preparation and Evaluation of Blank and PZQ-loaded PLGA NPs

As explained above, five main studies **(Figure 1.2)** were conducted to select the optimum or best level of each studied factor affecting the prepared NPs properties prepared by the single ESE method **(Figure 1.1)**. The PLGA NPs either plain or medicated were optimized with regards to their mean diameter, size distribution, charge, drug entrapment efficiency and drug loading.

They are also characterized for their surface morphology, thermal behavior, chemical integrity and drug release behavior. The results of each study will be discussed in this section.

II.B.1. Study I: Preliminary Phase

The first objective of this study was to choose the method of preparation of NPs loaded with PZQ **(Expt. I-1)**. Then, several process parameters were varied to identify their influence mainly on the particle size and entrapment efficiency keeping the other parameters constant as shown in **Table 1.1**. **Table 1.7** and **Figure 1.7** summarize the results of the eight different experiments **(Expt. I-1 to Expt. I-8)** conducted in this study. The selected level of each studied parameter was highlighted in yellow as shown in **Table 1.1**. The main findings will be highlighted and discussed as follow:

In **Expt. I-1**, three methods of preparation were used for formulation of PZQ- loaded PLGA NPs to study the **effect of organic solvent type**. Single (O/W) ESE method using DCM as organic solvent (Formula **P1**), ESD technique using the organic solvent EA (Formula **P2**) and nanoprecipitation method using acetone (Formula **P3**) were conducted.

Concerning formula **P3** prepared using acetone, it had the smallest particle diameter (96.71± 4.8nm) which is significantly lower than the particle size of both **P1** and **P2** (*p*-value <0.0001). However, its encapsulation efficiency is much more less than that of both formulae **P1** and **P2** (2.7 ± 1.13% in comparison to 4.2±0.85 and 4.15±0.99%, respectively). This could be due to the rapid formation of particles not allowing enough time for encapsulating more PZQ during the emulsification step; as the solvent used in nanoprecipitation method is partially miscible with aqueous phase leading to a decrease in the interfacial tension between the oil phase and aqueous phase with increasing its diffusion into the aqueous phase causing interfacial turbulence. This perturbation further results in a larger interface area between the oil droplets and aqueous medium and creates extremely fine nano- droplets which result in the formation of small sized nanoparticles rapidly. [79] On the other hand, formula **P2** prepared using EA as organic solvent showed the largest size measuring 341.83± 9.4nm which is significantly higher than that of formula **P1** (281.3±3.98nm).

The results also revealed that all the prepared NPs are negatively charged. This could be due to the presence of carboxylic groups on the surface of NPs especially that the PLGA selected

for NPs formulation was uncapped "acid-terminated" to enable its surface functionalization as will be explained in *Chapter Two*.[80] It was also noticed that the used levels of various parameters for preparation of PLGA NPs need further optimization to improve the particles' size distribution, reduce its diameter and increase the very low obtained EE% and DL%.

As a result, ESE method was used for the further preparation of PZQ- loaded PLGA NPs and the variables affecting it were studied to choose their optimum levels. As mentioned earlier, ESE technique has five successive phases: O/ W emulsification, DCM evaporation, NPs purification and washing, Pellet resuspension and lyophilization. The effect of variables controlling each phase was studied **(Expt. I-2 to I-8)** and the obtained results were shown in **Table 1.7** and illustrated in **Figure 1.7**.

It was noticed from **Expt. I-1** that the yield of formula **P1** was noticeably decreased after using rotavap for DCM evaporation to represent about 60.5± 4.1% of the initial added weights of polymer and drug. As a result, evaporation of the organic solvent under atmospheric conditions using magnetic stirrer (Formula **P5**) was compared to its evaporation under reduced pressure using rotating evaporator (Formula **P4**). **Effect of type of evaporation technique** was studied in **Expt. I-2**.

Comparing the two evaporation methods represented by formulae **P4** and **P5** respectively, there were no statistically significant differences in the physical properties of the prepared NPs. However, the EE% is decreased from 4.2±0.85% to 2.13±1.45%. But, this might be due to the long evaporation time on stirrer for about 12hr. Yet, the yield of NPs increased to reach 93.74 ± 2.33% in case of evaporation under atmospheric conditions.

Due to the results obtained from **Expt. I-2**, the **effect of the duration of evaporation** on NPs properties especially EE% was studied in **Expt. I-3**. Four time durations (2, 4, 6 and 12 hours) were tested. It was revealed that by reducing the time allowed for DCM evaporation, the EE% and DL% of PZQ inside PLGA NPs increases; and interestingly, the particles size decreases.

In a trial to increase the amount of the entrapped drug in NPs, the **effect of centrifugation conditions** for purification of formulated NPs and washing of excess SAA and un-entrapped PZQ from the surface of NPs was investigated (**Expt. I-4**). Formula **P11** showed

slight decrease in particle size (241.1±2.67nm) in comparison with 249.6± 3.33nm for formula **P10**. It also showed better size distribution (0.38±0.13 instead of 0.52±0.21) and slightly higher EE%. It was also noticed that the ultra- speed of centrifugation caused the aggregation of precipitated NPs and more difficult re-dispersion of the purified NPs pellet for further wash or use and it was taking long time vortexing.

The **effect of adding 0.01% L-leucine as antiaggregant** to the external aqueous phase in order to facilitate the NPs dispersion in ionized water after centrifugation was therefore tested (**Expt. I-5**). Formula P13 containing L-leucine showed lower PDI (0.29±0.11) than formula P12 (0.38±0.13).

The external energy applied for size reduction of the formed droplets in the emulsion to reach the nano- size could be of different sources: high speed homogenization, ultra- sonication or both. The **Effect of emulsification technique** was studied in **Expt. I-6**. Formula **P16** emulsified by homogenization followed by sonication reduced the size of nano-droplets in the emulsion producing homogenously distributed NPs population (0.07±0.09) when compared to formula **P14** (0.29±0.11) and **P15** (0.583±0.155).

Then, the **effect of sonication duration** was also studied **Expt. I-7**. In In this experiment, the effect of decreasing the sonication time on particles mean diameter, size distribution and the amount of drug encapsulated into NPs was investigated. By reducing the time duration of sonication, increase in the particles diameter and PDI was obviously noticed. The NPs size increased to 280.3±3.98nm in case of 5 minutes sonication (Formula **P19**) compared to 246.63±3.86nm and 222.97±2.09nm for formulae **P18** (10 minutes) and **P17** (20 minutes) respectively.

The last experiment in this phase was to study the **effect of freeze drying (Lyophilization**) of purified NPs on the various physical characteristics of PZQ- loaded PLGA NPs (**Expt. I-8**). No significant difference on characterization results were observed as shown by formulae **P20** and **P21** in **Table 1.7**. Similar findings were reported in previous studies. [65]

Generally, in all experiments in this study, no significant effect was noticed on surface charge of NPs. Zetapotential of all formulae ranged from -24 mV to -28 mV. The high negative

charge was due to the negativity of the used polymer in this study (Low molecular weight, uncapped PLGA 75:25). This could be explained by that, during preparation of PLGA NPs, oil droplets were formed in the aqueous medium consisting of the PLGA polymer encapsulating PZQ. This causes the ionizable carboxyl groups of the polymer to orient themselves towards the surface exerting the negative charge on the surface, while the hydrophobic portions formed the core of the polymerizing NPs matrix. This could be of benefit for the produced emulsion stability as the classic colloidal theory states that electrostatic forces at particle surfaces can cause repulsion and prevent aggregation by virtue of fewer collisions and ionic attractions. [81]

Also, there was no significant effect of the studied factors on PZQ encapsulation efficiency and in turn on its loading inside the polymer matrix. However, the resulted EE% and DL% were surprisingly extremely low (less than 5% and less than 1% respectively). This might be due to two factors; the first one is the low molecular weight of the used PLGA 75:25 (17000 Da) and the second one is its high hydrophobicity. It is worth mentioning that it was expected that such polymer properties should result in higher drug loading; since, PZQ was known to be highly hydrophobic in nature. However, by referring to the solubility properties of the drug [77], it was found that it has very slight solubility in aqueous solutions (0.22mg/ml). Accordingly, a less hydrophilic polymer with higher molecular weight (PLGA 50:50, 44000 Da) was used in **validation phase I** to study its effect on PZQ encapsulation efficiency and loading%.

II.B.2. Study II: Screening Phase

In the present study, the effect of variable parameters and conditions on the formulation of PLGA NPs loaded with PZQ was investigated: the PLGA concentration, the DCM volume and surfactant type in the formulation. Only one parameter was changed in each series of experiments. The impact of formulation variables were investigated mainly on size distribution, polydispersity and the surface charge of NPs. Three experiments were conducted in this phase. The studied variables and the obtained responses were summarized in **Table 1.8** and graphed in **Figure 1.8**.

Effect of Polymer Concentration was evaluated in **Expt. II-1**. Decreasing the PLGA polymer (% w/v) from 5% (Formula **S3**) to 2.5% (**S2**) and 1% (**S1**), though resulted in slight decrease in particle size, it had negative effect of size distribution as the PDI increased in case of formula **S1** to be 0.38±0.1 instead of 0.28±0.08 and 0.09±0.02 in case of formulae **S2** and **S3**

respectively. These results run with that published in previous studies which revealed that increasing the amount of polymer resulted in an increase in size of the formulation. [70,82,83]

In addition, the **effect of organic solvent %** was tested in **Expt. II-2**. While decreasing the volume of DCM resulted in significant increase in the particle size to be 290.3±3.75nm in case of 1% DCM (**S4**) compared to 225.77±0.92nm when 5% (**S6**) was used for preparation of PLGA NPs (*p*- value < 0.0001). It also increased the PDI to be 0.49±0.1.

Four types of SAA were used for formulation of PLGA NPs to evaluate the **effect of surfactant type** on NPs properties (**Expt. II-3**). Tween-80 resulted in statistically larger NPs size with heterogeneous size distribution of particles, Formula **S8** (545.97±7.3nm) compared to formula **S10** prepared using PVA (*p*- value < 0.0001). PVA, a non-ionic amphiphilic copolymer locates at the interface between particle surface and the aqueous medium during formulation. The hydrophobic segments of PVA penetrate and are entrapped in the polymeric matrix while the hydrophilic segments reduce interfacial tension. [81,85] Both poloxamer 188 (**S9**) and poloxamer 407 (**S7**) also increased the size and PDI of PLGA NPs significantly.

II.B.3. Study III: Validation Phase I

The selected levels of all studied variables in both studies **I** (preliminary phase) and **II** were collectively validated to see their simultaneous effect on NPs properties either as unloaded (Formula **VI1**) or PZQ- loaded (Formula **VI2**). Also, as the entrapped drug amount was still particularly low, PLGA having different characteristics (Higher molecular weight, uncapped PLGA 50:50) (**Table 1.3**) was used for the preparation of formulae (**VI3** and **VI4**). Results were demonstrated in **Table 1.9** and **Figure 1.9**. As shown, formula **VI4** prepared using PLGA 50:50 showed significantly higher encapsulation efficiency than formula **VI2**. Consequently, PLGA 50:50 was used for preparation of NPs in all the coming studies.

II.B.4. Study IV: Optimization Phase

The objective of this study was mainly to maximize the EE% and DL% while optimizing size, polydispersity and surface charge. In this study, PLGA NPs loaded with PZQ were formulated by emulsion solvent evaporation technique. The effect of various formulation variables as mentioned in **Table 1.4** were assessed systematically to obtain higher encapsulation

efficiency and drug loading and to minimize the NPs size. The impact of these variables on different NPs characteristics were recorded in **Table 1.10** and **Figure 1.10**.

As illustrated, **Expt. IV-1** tested the **effect of PVA concentration**. Decreasing the PVA%, although increased the particle size slightly, it increased the EE% and DL% by more than the double. Using 0.5% PVA (Formula **O1**) produced more homogenously distributed NPs (PDI = 0.04± 0.03) with PZQ EE% and DL% equal to 10.39±1.65% and 1.47±1.65%, respectively. This finding was in agreement with ones from other studies.[42,56,86]

As most of the parameters affecting the properties of PLGA NPs prepared by ESE method were studied and the EE% and DL% were still low. This was believed, unexpectedly, to be due to the very slight solubility of PZQ in the external aqueous phase. **Effect of salt addition to external aqueous phase** was studied in **Expt. IV-2**. Addition of salt to the external phase, which is completely soluble in the aqueous phase, was believed to reduce the solubility of the drug and, in turn, increase its encapsulation inside the PLGA polymer. The effect of two salts was assessed, sodium chloride and ammonium sulphate. In both types, the PZQ EE% increased significantly to be 27.22±1.17% and 26.33±1 for NaCl (Formula **O5**) and $(NH_4)_2SO_4$ (Formula **O6**), respectively. However, no significant difference between their effects on the physical properties of PLGA NPs, the physical stability of the O/W emulsion produced using $(NH_4)_2SO_4$ is much better than the one prepared by NaCl.

Based on the results obtained from (Expt. **IV-2**), higher concentrations of $(NH_4)_2SO_4$ were used to evaluate the **effect of salt concentration** on PLGA NPs properties (**Expt. IV-3**). Interestingly, 20% of salt in the external aqueous phase (Formula **O9**) increased the EE% significantly to reach 99.9±5% and the 10% salt concentration (Formula **O8**) resulted in EE% equals to 65.38±1.58%. Unfortunately, both concentrations affected the NPs size and distribution negatively. The size and PDI of formula **O8** were 610.1±6.56nm and 0.58±0.07, respectively. While, the size of formula **O9** was 608.2±4.7nm and PDI equals to 0.58±0.12. This could be due to a decrease in stability of the prepared emulsion as a result of reduced electrostatic repulsions upon addition of salts to the external aqueous phase causing flocculation of the particles due to the presence of positive Na^+ and NH_4^+ ions in the external phase. Therefore, 5% $(NH_4)_2SO_4$ was selected and further optimization was required.

The **effect of changing D:P** was studied in **Expt. IV-4**. Five ratios were tested ranging from 1:1 to 1:10. It was expected that increasing the polymer would result in higher EE%. Surprisingly, 1:10 (Formula **O14**), PZQ to PLGA ratio, resulted in significant decrease in EE% (3.65±5.43%) and DL% (0.331±5.43%) with sight increase in particles size (237.2±2.66nm). Interestingly, the 1:1 ratio (Formula **O10**) produced NPs with the highest EE% (37.9±2.1%) and DL% (12.63±2.1%) with slight decrease in size (193.6±1.89nm) and PDI (0.09±0.01). These findings were on the contrary of previously reported data that declared that the highest the polymer to drug ratio, the more entrapped drug would be. [53,70] The obtained results might indicate that the prepared PZQ- loaded PLGA NPs were in form of nanocapsules rather than nanospheres. **Figure 1.11** illustrated the difference in their structures; smaller amount of the polymer was required to encapsulate PZQ inside it rather than a large amount of PLGA where the drug molecules were being dispersed within. [53]

II.B.5. Study V: Validation Phase II

Selected levels of all tested variables in the four previous studies were validated for the preparation of the optimized formula either as blank or medicated PLGA NPs as shown in **Table 1.5**. Also, the **effect of initial drug weight** first dissolved in DCM was studied using two levels either 50mg or 100mg PZQ (**Expt. V-1** and **V-2**). The results obtained upon characterization were shown in **Table 1.11** and illustrated in **Figure 1.12**. It was clear that combining all the selected levels together resulted in further increase in EE% and DL%. Formula **VII2** increased the PZQ encapsulation to be 77.16±2.05% with drug loading equals to 38.59±2.05%. Further increase in EE% and DL% was achieved when the initial drug weight used for preparation of NPs was increased to be 100mg instead of 50mg. Formula **VII4** showed NPs with about 94.19±3.46% of encapsulated PZQ which was equivalent to 47.095±3.46% of PZQ loading. Consequently, formula **VII4** was selected as the optimized formula for further characterization and experimentation. A second formula (**VII8**) was suggested as the second optimized formula. As shown in Table I11, the blank formulae (**VII1**, **VII3**, **VII5** and **VII7**) were slightly smaller in size than the medicated formulations.

II.C. Further Characterization of Selected Optimum Formulation

By the optimization of formulation process, the PZQ- loaded PLGA NPs optimum formula **VII4** were obtained in submicron size from 200.8 ± 4.02 nm and monodispersed (PDI

0.13 ±0.08) . The encapsulation efficiency of nanoparticles loaded with PZQ was 94.19 ± 3.46 % and drug loading equals to 47.095 ±3.46%. This selected formula was further observed by SEM and AFM. NPs presented smooth surface and spherical shape. Also, their physical properties and thermal behavior was characterized using DSC and their chemical integrity was evaluated using FTIR. Furthermore, lyophilized NPs was evaluated for *in vitro* release in phosphate buffered saline (pH = 7.4) by using dialysis bag diffusion technique.

II.C.1. Thermal Analysis Using Differential Scanning Calorimetry "DSC"

To further assess possible interactions between PZQ and the polymer, DSC analysis was performed on pure PZQ and PZQ- loaded nanoparticles. **Figure 1.13** shows DSC thermogram that helps to know the nature of the encapsulated drug in the NPs as the physical state of the drug in the polymeric matrix could influence its release characteristics.

It was observed that the endothermic peak of PLGA is at 54^{o}C as PLGA exhibited a glass transition temperature (Tg) at 54^{o}C. The endothermic peak of pure PZQ was found approximately at 136^{o}C. This characteristic peak was not observed in PZQ- loaded NPs. The absence of detectable crystalline domains of PZQ in drug loaded NPs clearly indicates that PZQ encapsulated in NPs is in the amorphous or disordered-crystalline phase or in the solid-state solubilized form in the polymeric matrix. This disordered- crystalline phase of PZQ inside the polymeric matrix helps in sustained release of the drug from the NPs. Thermogram of the physical mixtures of PLGA and PZQ (1:1) or PVA and PZQ (1:1) are superimposition of the curves of their singles. Absence of the PVA characteristic peak from the curve of PZQ- loaded PLGA NPs confirms its thorough washing while purification of NPs.[68,87,88]

II.C.2. Spectral Analysis Using Fourier-Transform Infrared Spectroscopy "FTIR"

FTIR uses infrared radiation to determine the chemical functionalities present in a sample. When an infrared (IR) beam hits a sample, chemical bonds stretch, contract, and bend, causing it to absorb IR radiation in a defined wavenumber. The resulting plot is of absorbance (or transmittance) versus wavenumber as shown in **Figure 1.14**. As it can be seen, free PLGA ester and acid bonds absorption peaks can be observed in 1760 cm^{-1} and 1713cm^{-1}. While, free PZQ's carbonyl functional groups was stretching at 1630cm^{-1}. Another stretching vibration caused by – C—N appeared from 1350 – 1000cm^{-1}. **Figure 1.15** shows the chemical structure of

PZQ. The physical mixture and NPs showed an overlap between the two infrared curves of PLGA and PZQ. This induced that there's no strong chemical interaction between PLGA and PZQ. [68,74,84,89]

II.C.3. *In Vitro* Release Study

Efficient release of encapsulated drug from NPs is an important parameter for developing successful formulations. The obtained results in **Table 1.12** and **Figure 1.16** clarified the release pattern of PZQ from PLGA NPs (Optimum Formula **VII4**) compared to PZQ alone (Control) over 35 days. It was evident that more than 90% of PZQ powder was dissolved and diffused through the dialysis membrane in the first 24 hours (93.77±2.02%) compared to only 54.21±2.88% in case of PZQ- loaded PLGA NPs (Initial burst release). The high initial burst release could be due to easy diffusion of drug molecules through the polymeric matrix at the beginning followed by a sustained release of the encapsulated drug. This finding was further supported by the thermal behavior of PZQ- loaded PLGA NPs studied using DSC. As previously mentioned, the presence of the drug in amorphous or in disordered-crystalline phase would help it to diffuse through the small pores of the NPs easier than if the drug was in crystalline form (large sized molecules) inside the particles. [48]

Then, during the first 2 weeks of the study, slow sustained release of PZQ was observed. The curve was almost a plateau with the release of about 62.46±2.74%. This might be due to diffusion of the drug through PLGA polymer matrix. Especially that the pH of the release medium was still ranging from 7.2 to 7.4 which means that PLGA was not yet started to degrade to its monomers, lactic and glycolic acids (**Figure 1.17**).[53,90,91] However, after 14 days, sustained release was continued though on a higher rate. By measuring the pH of the release medium, it started to decrease till reaching pH equivalent to 6.1 at the end of study duration. This means that the release of PZQ was started to be due to the degradation of the PLGA polymer in a very slow rate as well. The amount released by the end of the 35 days was 79.82±2.45%; this confirms that sustained release of PZQ from PLGA NPs was successfully achieved.

II.C.4. Particle Morphology

Morphology of prepared PLGA NPs was characterized using different techniques: optical microscope, scanning electron microscopy (SEM) and atomic force microscopy (AFM).

II.C.4.1. Optical Microscope

Figure 1.18 shows PZQ- loaded PLGA NPS prepared by ESE using optimum levels (Formula **VII4**). Spherical shape and homogenous particle size distribution were evident.

II.C.4.2. Scanning Electron Microscopy

When a sample is bombarded with electrons, it emits secondary electrons and X-rays. The intensity of the secondary electrons is detected to generate a high resolution three dimensional surface image. X-rays can be detected to conduct elemental analysis. SEM is not as surface sensitive as other techniques and non- conducting polymers must be sputter-coated prior to analysis. Nevertheless, it is one of the more widely available tools in surface analysis, and it is thus often used to measure surface topography.[92] **Figure 1.19** is a scanning electron micrograph of formula **VII4** showing spherical NPs with smooth surface. **Figure 1.20** shows scanning electron micrographs of various prepared formulae.

II.C.4.3. Atomic Force Microscopy

AFM has sub-nanometer resolution. In AFM, a tip attached to a flexible cantilever moves across the sample surface to measure surface morphology on an atomic scale. The change in surface height is then measured by the location of the reflected laser beam in the quadrant photo-detector, and surface topography is generated.[93] **Figure 1.21** shows PLGA NPs of formula **VII4** with smooth surface and spherical morphology**.**

Chapter Two: Development of Immuno—Nanoparticles for Active Targeting of Schistosoma Parasites

Section I: Methodology[*]

[*] **All materials and equipment used in this study were described in *Appendix I* and *Appendix II* respectively**

The aim of the work presented in this chapter is to prepare antibody- coated PLGA NPs for active targeting of *Schistosoma* parasites. To achieve the set objective, the work was divided to two main sections. The first one is the production, purification and characterization of polyclonal antibodies "pAb" specific against schistosomes' surface antigens "sAg". While the second study was the surface functionalization and coating of the optimized PZQ-loaded PLGA NPs formula (**VII4**), prepared in *Chapter One*, with the purified pAb

Section IA: Preparation of Anti-schistosomal Polyclonal Antibodies against *Schistosoma* Surface Tegumental Antigens

To prepare the anti- schistosomal pAb, we first prepared the *Schistosoma* sAg to immunize the rabbits so that they can produce specific pAb against it.

I.A.1. Production, Purification and Characterization of Tegumental *Schistosoma* Antigens "sAg"

I.A.1.1. Production of *Schistosoma* sAg

Viable *Schistosoma haematobium (*Egyptian strain) worms were obtained from the Schistosome Biological Supply Center (SBSC), Theodore Bilharz Research Institute (TBRI), Giza, Egypt directly after their perfusion from the porto- mesenteric veins of infected golden hamsters. The collected mature worms were washed three times with sterile PBS (pH 7.4). The crude excretory/ secretory (E/S) *S. haematobium* antigens were prepared by incubation of the parasites overnight in Roswell Park Memorial Institute (RPMI-1640) culture medium, maintained at pH 7.4 with HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)

20mM, and supplemented with l-glutamine, 10% Fetal Bovine Serum (FBS) and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin and 160µg/ml gentamycin) at 37^{o}C under 5% CO_2 in air.

Following incubation, the medium is removed and centrifuged at14000 rpm for 30 min at 4^{o}C. The collected supernatant, containing **the excretory-secretory (E/S) crude antigens**, is aliquoted and stored at -20^{o}C till use. [94,95,95–97]

I.A.1.2. Purification of *Schistosoma* sAg

Afterwards, **Cysteine proteinases** (CP Ag), molecular weight (M.Wt.) of 27-29 kDa, were purified from the crude extract by a combination of ion exchange chromatography on DEAE (diethyl amino-ethyl) -Sephadex A-50 column and gel filtration using Sephacryl S-100 HR column.

I.A.1.2.1. Ion Exchange Chromatography

Ion exchange chromatography is the most popular technique for purification of proteins based on their net charge. Herein, we purified the prepared crude antigens using DEAE chromatography. The DEAE group maintains a constant positive charge that is neutralized by counter ions, usually chloride ions. Other anions are capable of competing for the positive DEAE group.[98] **Figure 2.1** is an illustration of the basic principle of ion exchange chromatography.

First, one gram Sephadex A-50 powder was swelled in about 200 ml of 0.5M Tris - HCI buffer (pH 7.4). Gentle stirring using glass rod during slow addition of the powder to the buffer was necessary as vigorous stirring might damage the particles. Complete gel swelling took 2 hours at 100˚C in water bath. The high temperature serves to deaerate the medium. After swelling, the initial supernatant was removed and the swelled gel was washed extensively with 20mM Tris-HCl buffer (PH 6.5), the selected binding buffer. The added 1gm Sephadex A-50 powder was swelled to 22 ml beads. Then, the swelled beads suspension (slurry with 75% settled beads and 25% buffer) was poured in 30x2.5 cm column in one continuous motion down a glass rod held against the wall of the column to avoid air bubbles trapping.

Following the settling of beads in the column, the surface was covered with the binding buffer and the approximate column binding capacity was determined. Since the available

capacity of DEAE -Sephadex A-50 is 5 gm protein/1g powder so, the column binding capacity was found to be equivalent to about 227mg protein per 1 ml swelled beads.

On the other hand, the protein sample (Crude Ag) was dialyzed versus the binding buffer and its protein content was calculated. The outlet tubing of the column was then closed and the buffer above the beads was removed. The protein content < 10% of column bed capacity (i.e. mass of beads inside the column) was pipetted to the column. The outlet tubing was opened till the sample penetration to the beads then closed again for 10 minutes to allow for protein adsorption to the beads via the ionic interaction between the oppositely charged ionic groups in the sample proteins and in the functional ligand on the beads. The outlet was then opened and the beads were washed by 5 bed volumes binding buffer.

The protein was eluted by 20mM Tris-HCl/150 mM NaCl under gravity by collecting 2ml fractions. By using high concentration salt as eluting buffer, the molecules with the weakest ionic interactions started to elute from the column first then the ones that have a stronger ionic interaction eluted later. The Absorbance of each fraction was measured at 280 nm and the purity of the produced protein was assayed by SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis). [94,98–100]

I.A.1.2.2. Gel Filtration (Size Exclusion) Chromatography

The fraction that showed the highest absorbance reading at 280nm, meaning that it contains the highest protein content, was further purified using gel filtration chromatography. This type of chromatography has been used for further separation of proteins, this time, based on their molecular size. The solid phase matrix (Sephacryl S-100 HR beads) consists of porous beads that are packed into a column with a mobile liquid phase flowing through the column. These porous beads act as sieves to filter small molecules which become temporarily trapped within the pores allowing enough time for large molecules to be pass first from around "be excluded from" the beads. Thus, protein molecules of different size will elute through the stationary phase "beads" at different rates and collected as fractions. **Figure 2.2** is a representation of the principle of size exclusion chromatography.

Briefly, the column was prepared by suspending Sephacryl S-100 HR powder in deionized water. Complete swelling was achieved after 5 hours boiling in water bath. The

swelled beads (50% settled medium) were poured in 30x2.5 cm column, avoiding air bubbles trapping as mentioned before, and washed with 3 column volumes mobile phase and left to be packed for about 24 hours. The outlet of the column was opened for mobile phase penetration in the gel surface. The sample (4% of the column volume) was layered on the bed surface and left to penetrate in the beads then the beads were washed by 4 bed volumes of mobile phase. Fractions (2ml each) were collected under gravity and absorbance at 280 nm of each fraction was measured. The fraction that showed the highest absorbance value was then analyzed by SDS-PAGE under reducing conditions.[94,96,98]

I.A.1.3. Characterization of *Schistosoma* sAg

I.A.1.3.1. Protein Content Determination

Concentrations of prepared crude *Schistosoma* antigen and the purified protein were determined using Bradford protein quantification technique. This assay is based on the change in color of the Coomassie dye from blue to reddish brown in direct proportional response to the various concentrations of bound proteins as this dye has high affinity for protein binding.[101–104]

Briefly, the dye was diluted with 4 volumes of deionized water and 200µl from the diluted dye were added to each dilution from the standard and the unknown protein samples in 96-well plate. The absorbance of the color was measured at 595 nm. Serial dilutions of initial known concentration of bovine serum albumin (BSA) were prepared to reach final concentrations range from 0 to 1500µg/ml. Consequently, a standard curve was constructed by plotting the BSA concentration versus absorbance. From the standard curve, the protein content of unknown samples was calculated. All experiments were carried out as triplicates.

I.A.1.3.2. UV Spectrophotometry

The absorbance of each eluted fraction from both ion exchange and gel filtration columns was monitored at 280 nm using a UV spectrophotometer. The cut-off absorbance was an optical density (OD) of 0.05. The fractions containing the specific component with an approximate molecular weight (M.Wt) of 27-29 kDa (CP Ag) were identified by SDS-PAGE.[94,98]

I.A.1.3.3. Molecular Weight Characterization

Purity of 27-29 KDa *S. haematobium* CP AG was assessed using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis **(**SDS-PAGE) technique. This technique is based on migration of proteins depending on their charge and size. However, in the presence of sodium dodecyl sulfate (SDS), all proteins become negatively charged owing to their binding to negatively charged detergent molecules. When these SDS coated proteins are placed in an electric field, the separation of the proteins will depend only on their size. [94,99,100]

The protein fractions of interest were analyzed by SDS-PAGE under reducing conditions on a 12 % polyacrylamide gel. For molecular weight estimation, low range pre-stained molecular weight standards from 18.5 to 106 kDa were diluted in sample buffer 1:20 (v/v). The prepared samples and low molecular weight standard were applied into the wells of the prepared gel (20 µl/well for sample and 10 µl for standard) and ran in parallel. After electrophoresis, the resolved proteins were revealed by staining with Coomassie brilliant blue dye.

The slab gel mold **(Figure 2.3)** was prepared within two glass plates separated by spacer bars and stood vertically into a glass cassette. The gel was prepared starting by the separating gel (12%) (Small pore gel) in the bottom of the slide and the stacking gel 4% (large pore gel) was poured on top to form the upper gel acrylamide. A plastic comb (1mm) was inserted into the stacking gel to form the sample wells and left to polymerize for 1 hr.

After polymerization, the comb was removed gently and the slab cassette inserted into the electrophoresis cell which was filled with the electrode buffer. The current was turned on and the run was started by 10 mA until the samples entered the stacking gel, and then the current was raised to 50mA until the samples reached the bottom of the gel. After electrophoresis, the gel was stained in 0.1% Coomassie blue (w/v) in methanol, acetic acid and water as 4:1:5 (v/v/v) for 2-4 h , then the excess dye was removed from the gel by destaining solution to visualize the protein bands. After destaining the gel was stored in 5% acetic acid.

I.A.1.3.4. Assessment of reactivity and specificity of the prepared *S. haematobium* CP antigen by indirect ELISA

To assess the reactivity of the purified S. haematobium CP Ag and its specificity to anti-S. haematobium immunoglobulins (Antibodies), indirect Enzyme-linked Immuno-Sorbent Assay

(ELISA) was carried out. **Figure 2.4** is a graphical representation of its basic principle. Briefly, ELISA test involves the passive adsorption of antigen onto the surface of solid phase e.g. microtiter plate followed by addition of serum containing specific antibodies. Indirect method involves incubation of the coated antigen with unlabeled antibodies (primary) then enzyme labeled antibodies specific to the primary one (indirect ELISA). When a suitable substrate is added, hydrolysis occurs, the degree of which is proportional to the amount of attached enzyme. The enzyme and the substrate are chosen to produce colored enzyme-substrate breakdown. **94,96,98,105**

Wells of polystyrene microtiter 96- flat bottomed wells plates were coated with 1µg/well of *S. haematobium* CP Ag in coating buffer (0.05 M carbonate buffer, pH 9.6), then incubated overnight at 4°C. The plates were washed 3 times with washing buffer 0.1 M PBS pH 7.4, and then blocked with 200 µl/well of 0.1% bovine serum albumin (BSA) in 0.1 M PBS tween-20 (PBS/T) pH 7.2 for 1 hour at 37°C. Following washing the wells 3 times, 100µl/well of diluted serum samples (1:200) containing primary antibodies diluted in the washing buffer was added and then incubated for 1hr at 37°C. Three serum samples containing specific antibodies against Schistosomiasis, Fascioliasis and Ascariasis (To detect for the specificity of tested *Schistosoma* Ag towards Anti- *Schistosoma* Ab) were used.

Then, the plates were washed 3 times with the washing buffer and 100µl/well of the diluted horse- radish peroxidase-labeled conjugate (labeled secondary antibody) was applied with incubation for 30min at 37°C. After 3 times washing, 100µl from the freshly prepared Ortho-phenylenediamine dihydrochloride (OPD) substrate solution in 0.05 M. Phosphate citrate buffer pH 5 was added to each well. The plates were incubated in the dark at room temperature for 30 minutes till color appearance. After that, stopping buffer (8 N H_2SO_4) 50µl/well was added to stop the over enzyme-substrate reaction. The absorbance was measured at 492 nm using ELISA reader.

The data were presented as mean ±SD. The means of the different groups were compared using the analysis of variance ANOVA. The data were considered significant if P values < 0.05.

I.A.2. Production, Purification and Characterization of Anti-schistosomal Polyclonal Antibodies "pAb"

I.A.2.1. Production of Anti-schistosomal pAb*

* *Experiment **I.A.2.1** was done by Dr. Ibrahim Rabie, Professor of Parasitology, TBRI*

In brief, two Newzealand white male rabbits, weighing approximately 3 Kg and about 4 months age, were examined before the immunization with the previously purified *Schistosoma* CP Ag for production of anti- *Schistosoma* polyclonal antibodies (pAb). They were free from *Schistosoma* and other parasitic infections. Then, they maintained at the SBSC/TBRI, Giza, Egypt. They were kept under standard laboratory care (Temperature 25˚C, humidity 45-55%, filtered drinking water, and 24% protein and 4% fat diet). Animal experiments have been carried out according to the internationally valid guidelines and ethical conditions. [100,106]

For immunization of the rabbits, 1 mg of CP antigen was mixed with an equal volume of complete Freund's adjuvant (CFA) and injected intramuscularly into each rabbit. Booster doses (0.5 mg mixed with an equal volume of incomplete Freund's adjuvant (IFA)) were administered at week 2, 3 and 4 after the initial dose. Sera were collected 4 days post last injection from ear vein. [100,107]

Blood samples were withdrawn from the rabbit's ear before the first intramuscular injection and examined by **indirect ELISA** for *cross reaction* with other parasite (to make sure that the rabbit was clean from any infection with other parasites) and after injection of each immunizing dose (boosting) to detect the titer of *specific anti-S. haematobium antibodies* produced. When the titer was high approximately the fourth day post last injection), the animal was scarified and blood samples were collected and antisera were further purified as explained below. [106,107]

I.A.2.2. Purification of Anti-schistosomal pAb

The collected rabbit serum containing the anti- Schistosoma pAb were purified on three successive steps. First, ammonium sulphate precipitation method was used followed by Caprylic acid purification technique. Finally, the partially purified protein is separated on DEAE anion exchange chromatographic column.

I.A.2.2.1. Ammonium Sulfate Precipitation

The principle of this method is based on the fact that proteins in solutions form hydrogen bonds with water through their exposed polar and ionic groups. When high concentration of highly charged ions such as ammonium or sulfate is added, these groups compete with the proteins for binding to water. This removes the water molecules from the proteins and decreases its solubility, resulting in their precipitation. [98]

Briefly, saturated ammonium sulfate solution was prepared by dissolving 100 g of ammonium sulfate in 100 ml deionized water, at 50^{o}C and kept overnight at room temperature. After complete dissolving, the supernatant was separated and the pH was adjusted to 7.4 using concentrated ammonia. On the other hand, Anti–*CP* polyclonal antibodies were centrifuged at 11000rpm in cooling centrifuge at 4^{o}C for 20 minutes to remove any debris.

The saturated solution was then added drop wise to anti–*CP* polyclonal antibodies to reach 50 % saturation, with continuous stirring. Afterwards, centrifugation at the same conditions was done. Supernatant was discarded. The precipitate was dissolved in a suitable amount of 0.01 M PBS, pH 7.4. The ammonium sulfate was removed by dialysis against 0.01 M PBS pH 7.4 for 72 days at 4^{o}C.

I.A.2.2.2. Caprylic Acid Purification Method

The principle of this method is based on the fact that the addition of short-chain fatty acids such as caprylic acid in mildly-acidic conditions to the serum. will precipitate most serum proteins with the exception of immunoglobulin G (IgG), which is the protein developed in response to Rabbit immunization with the Schistosoma CP antigen. [98]

Briefly, the Anti–CP polyclonal antibodies, partially purified by ammonium sulfate precipitation were diluted with 2 volumes of 60 mM sodium acetate buffer pH 4.8. Then, 7% caprylic acid was added drop wise with slow magnetic stirring for 30 minutes at 4^{o}C. The mixture was then centrifuged at 11000rpm for 30 minutes at 4^{o}C; serum proteins except IgG were separated and removed in the precipitate while the supernatant, containing nearly pure IgG, was further purified.

I.A.2.2.3. Ion Exchange Chromatography

The same technique that was demonstrated under subsection (**I.A.1.2.1)** was repeated. However, the purified anti- CP IgG sample was applied to the column. Upon fractionation, the absorbance of each collected fraction was measured at 280 nm via **UV Spectrophotometry**.

The fraction exhibiting the highest absorbance (The purified anti- *Schistosoma* pAb) was further characterized for its protein content (**Bradford Protein Quantification Assay**), purity and molecular size (**SDS-PAGE**), and finally, its specificity against *S. haematobium* CP Ag (**Indirect ELISA**).

Section IB: Preparation and Characterization of PLGA Nanoparticles Surface – Modified With Purified Anti- *Schistosoma* Polyclonal Antibodies

The aim of the study was to functionalize the surface of the optimized PZQ- loaded PLGA NPs formula (**VII4**) with the purified anti- *Schistosoma* pAb in order to achieve a specific drug carrier system against schistosome surface antigens. Presumably, when such a drug delivery system (DDS) is administered systemically, it will help for active targeting of the drug towards *Schistosoma* parasites in host blood circulation. **Table 2.1** summarizes the properties of the chosen optimum formula and its blank.

I.B.1. Preparation of Polyclonal Antibody- Coated PLGA NPs "Immuno-NPs"

Two methods for coating the surface of PLGA NPs were studied. The first one is the non-covalent physical adsorption of the pAb to the NPs' surface and the second method is the covalent conjugation through carbodiimide chemistry. The method for the preparation of immuno-NPs was optimized on the basis of pAb- coupling efficiency, the amount of adsorbed or conjugated pAb to PLGA NPs (%w/w) and the reactivity of the coupled pAb against *Schistosoma* CP Ag. The prepared immuno-NPs were also characterized for their size, size distribution, charge, drug encapsulation and drug loading in order to assess the effect of coating method on the properties of the optimized PLGA NPs.

I.B.1.1. Physical (Non- Covalent) Adsorption Method

The prepared PLGA NPs (0.5 mg/ml) were first dispersed in PBS, pH 5.5, by sonication for 20 seconds. Specified volumes of the solution containing the purified pAb (2 mg/ml) were then added to the NPs' suspensions and the volumes were adjusted using PBS, pH 5.5 to reach

the final specified ratios mentioned in **Table 2.2** (**Experiment I-2:** Studying the effect of the amount of added pAb "pAb:PLGA (%w/w)" on adsorption efficiency**)** . The mixtures were mixed very well by vortexing at room temperature then, left for either 12 hours or 24 hours (**Experiment I-1**: Studying the effect of incubation time on adsorption efficiency, **Table 2.2**) at 4^{o}C to allow for pAb adsorption on the surface of PLGA NPs. After that the suspension was centrifuged for 15 minutes at 11000rpm in a cooling centrifuge to separate immuno-NPs from free pAb. The precipitate was washed twice for 10 minutes with PBS, pH 7.4 then, re-suspended in the same buffer for further use. The supernatant from both purification and washing centrifugation steps was collected for quantification of the free unattached pAb by Bradford protein quantification method. The control was run the same way as the sample but PBS was used instead of pAb solution as illustrated in **Table 2.2**.[108,109]

I.B.1.2. Chemical (Covalent) Conjugation Method

For covalent attachment of anti- CP pAb onto the PLGA NPs surface, EDC/NHS carbodiimide chemistry was employed.[110–113] Briefly, PZQ- loaded PLGA NPs (0.5mg/ml) were dispersed in PBS, pH 7.4. Then, carboxylic groups (-COOH) were activated prior to their incubation with antibody by addition of the water-soluble 1-ethyl- 3-(3-dimethylaminopropyl) carbodiimide (EDC), 2mM and 5mM N- hydroxysuccinamide (NHS) in PBS, pH 5.5 to the suspension. The sample was stirred at RT 1 h on a rotating wheel. Then, the sample was centrifuged at 11000 rpm, 4^{o}C for 15 min to remove unreacted EDC and NHS. The process was repeated twice and the precipitated particles were washed each time with PBS, pH 7.4.

Afterwards, the pellet obtained after washing was suspended in PBS, pH 7.4 and a buffered solution containing the determined amount of pAb was added (**Table 2.3**, **Experiment II-1:** Studying the effect of the amount of added pAb "pAb:PLGA (%w/w)" on conjugation efficiency). The mixture was rotated for 1hr at RT before overnight incubation at 4^{o}C. Then, the formulated immuno-NPs were purified and collected by centrifugation three times at 11000 rpm, 4^{o}C for 20 min to remove any excess unconjugated pAb. The final precipitated pellet was suspended in PBS pH 7.4 for further experiment. The supernatant was collected during the washing steps to measure the amount of unconjugated pAb spectrophotometrically at 595 nm using ELISA plate reader. Again, the control was run the same way as the sample but PBS was used instead of pAb solution as illustrated in **Table 2.3**.[48] [acharya2009targeted][48] [

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I.B.1.3. Statistical Analysis

All experiments were carried out three times, independently. Results are expressed as mean ± standard deviation (SD). Significant statistical differences between groups were examined by applying Student's t-test for two groups' comparison and one-way analysis of variance (ANOVA) for three or more groups. The statistical significance level (*P-value*) was set at ≤0.05. Statistical analysis was done to all data obtained for all NPs formulae. All tests were calculated using the software ***GraphPad Prism Software Version 6, San Diego, CA***.

I.B.2. Characterization of Polyclonal Antibody- Coated PLGA NPs

All prepared formulations of both pAb- adsorbed and pAb-conjugated PZQ-loaded PLGA NPs were characterized on basis of their **size**, **size distribution**, **charge** and **drug encapsulation** (Refer to methodology section in ***Chapter One***) to assess the effect of coating methods on the optimized properties of PLGA NPs. In addition, the amount of attached pAb to the surface of PLGA NPs was quantified and the **adsorption and conjugation efficiencies** were calculated. Accordingly, the optimum immuno- NPs formulae were selected and the **reactivity of pAb,** after being attached to NPs, against *S. haematobium* CP Ag was assessed using dot blot immuno- assay.

I.B.2.1. Quantification of pAb on Surface of PLGA NPs

The amount of attached pAb on the surface of PLGA NPs was determined using Bradford protein quantification assay (Refer to subsection **I.A.1.3.1.**, ***Chapter Two***). An indirect method was used where the pAb concentration was quantified in the supernatant collected during the purification and washing steps of the prepared immuno-NPs. Then, the actual attached pAb concentration was calculated, based on the constructed BSA standard curve, by subtracting the measured concentration from the initial used one. All analysis was performed in triplicate. [64,113,114]

I.B.2.2. Bioactivity Assay

This experiment was done to test for the reactivity of pAb against sAg after being adsorbed or conjugated to the surface of NPs; as it has been known that attaching the bioactive

molecules, particularly via chemical conjugation, often reduces their activities. [75] The bioactivity of pAb- conjugated PLGA NPs was assessed qualitatively using modified dot blot immuno-assay and compared to that of free pAb (Positive control). [115–117]

An indirect type immuno-assay (**Figure 2.5**) modified approach was successfully developed for pAb-PLGA NPs bioactivity evaluation. Concisely, an identified amount of *S. haematobium* purified CP Ag (1µg), diluted in 1X Tris- buffer saline (TBS, pH 7.4), are spotted on to wet nitrocellulose membrane and left to dry for 1hr at RT. Upon that active sites on the membrane were blocked using 3%w/v skimmed milk powder for 1 h at 37^{o}C with gentle agitation followed by washing thrice in TBS – 0.2% Tween 20 (TBS-T), pH 7.4.

After washing, immuno-NPs surface coated with purified anti-CP pAb via carbodiimide conjugation reaction, Formula **C5** in concentration equivalent to 1µg pAb in TBS-T buffuer and its Blank (Formula **C1**) were pipetted over previously spotted sAg on the membrane. Purified CP antibody (Primary antibody) alone was applied to serve as positive control for the experiment. On the other hand, negative control was run in parallel by adding buffer free of antibody or immuno-NPs. Incubation at 4^{o}C was followed overnight to allow enough time for the formation of sAg-pAb complexes. Unconjugated primary antibody was washed away with TBS, pH 7.4 thrice.

The formed complexes were detected through reaction with enzyme- linked secondary antibody "Horse Raddish Peroxidase (HRP) conjugated anti-rabbit secondary antibody at concentration (1/10000 dilution)" and incubated for 1 h at 37^{o}C. In turn, unbound secondary antibody as well was removed by washing. Washed membranes were then incubated with soluble uncolored TMB liquid substrate diluted in TBS and left to develop the color for 10 minutes. The enzyme-substrate conjugate was converted to an insoluble purple product by the enzyme. The intensity of the developed color was used as an indication of activity of antibody present in the sample. Finally, the membranes were dried and scanned. Each control and sample was carried out in triplicates.

Section II: Results and Discussion

Section IIA: Preparation of Anti-schistosomal Polyclonal Antibodies against *Schistosoma* Tegumental Antigens

In this study, *S. haematobium* parasites were collected from the porto- mesenteric veins of infected hamsters and the E/S crude antigen was extracted from the worms as explained earlier. The crude sAg was then purified on two steps using ion exchange and size exclusion column chromatography methods. The purified CP antigen was further characterized to evaluate its protein content, molecular size, bioactivity and specificity against anti- CP antibody.

Figure 2.6 shows the OD_{280} profile of the antigen fractions obtained following purification of E/S products of adult *S. haematobium* worm by **DEAE Sephadex A-50 ion exchange chromatography** using **UV spectrophotmetry**. The eluted antigen is represented by a single peak with maximum OD value equal to 2.64 at fraction number 11 representing the eluted antigen (the fraction with the highest protein content). The eluted proteins from the DEAE-Sephadex A50- ion exchange column chromatography were analyzed by **12.5% SDS-PAGE under reducing conditions** (**Figure 2.9**), showing several bands of different molecular sizes representing the cysteine proteinase antigen along with other proteins. The band showed at 27-29 kDa was the one of interest representing the CP antigen.

The partially purified Cysteine proteinase antigen obtained following purification by DEAE Sephadex A-50 ion exchange chromatography (Fraction number 11) was further purified by **Sephacryl S-100 HR gel filtration column chromatography** and one peak was obtained representing the column elution volume fractions which contain the highest protein content with OD value 1.91 at fraction number 7 (**Figure 2.7**). The eluted proteins from the gel filtration column chromatography, analyzed by **12.5% SDS-PAGE under reducing condition** showed only one band at 27.5 kDa which was cysteine proteinase antigen (**Figure 2.9**).

For protein content determination, **Bradford protein assay** was performed. First, BSA (Bovine serum albumin) standard curve was established. Serial dilutions from BSA stock

solution of known concentration were prepared (**Table 2.4**). The assay was conducted as explained under the methodology section and the absorbance was measured for the different dilutions at 595nm. Concentrations were plotted versus absorbance and a calibration curve was generated, as shown in **Figure 2.8**. Polynomial equation (R^2 = 0.999) was obtained and used for calculations of protein concentration. It is worth mentioning that the polynomial equation resulted in more accurate calculations of protein concentration, even though several trials were done for linearization of Bradford protein assay. [101,102,104,114] Two ranges of serially diluted BSA concentrations were used in order to achieve more accurate determination of protein content at very low concentrations (0-25µg/ml), as illustrated it **Table 2.4b** and **Figure 2.8b**.

Then, the total protein content of the E/S crude antigen products of *S. haematobium*was measured. It contained 2.7±0.045 mg/ml of total protein. While, after purification, the final protein content of purified *S. haematobium* CP antigen was 1.85±0.071 mg/ml.

Furthermore, to confirm the purity of the 27-29 kDa separated fraction (Purified CP sAg), the eluted proteins gained from the different purification steps were analyzed by 12% **SDS-PAGE** under reducing conditions. **Figure 2.9** shows the gel profile of adult *S. haematobium* worm antigens (The crude E/S sAg before purification, the partially purified CP sAg after ion exchange column chromatography and the purified CP sAg). Low molecular weight of standarad proteins was run in parallel showing different bands ranged from 18.5 -106 kDa to help estimate the molecular size of the eluted proteins. Stained gels showed that the crude antigen productss was separated in multiple bands of M.Wt. ranging from 12-117 kDa with major bands of 105, 79, 66, 58, 45, 34, 29, 27 and 14 kDa and minor bands of 100, 38, 36, 32, 25 and 12 kDa. On the other hand, SDS-PAGE profile of the purified fraction showed a single homogenous band of 27-29 kDa. The 27-29 kDa molecules have been reported earlier as the *S. haematobium* CP enzyme (ShCP3) based on its similarities to a 28 kDa CP from *S. mansoni*. [106] Based on this, we suggested that our purified sAg might be the *S. haematobium* CP reported earlier.

Reactivity and antigenicity of the purified target CP antigen was evaluated by **indirect ELISA** technique. Herein, serum samples from *S. haematobium* infected rabbits gave a strong reaction against *S. haematobium* CP products with absorbance reading equal to 1.41 ± 0.09. While, no cross reactivity was recorded with sera of rabbits infected with other parasites e.g.,

fascioliasis, and ascariasis (**Table 2.5**) as the absorbance reading for *S. haematobium* was significantly higher than the ones obtained from the sera containing pAb against other parasite infections. This confirms the specifity of our CP sAg against *S. haematobium* pAb.

Consequently, male New Zealand white **rabbits were immunized with the purified *S. haematobium* CP antigen** and the reactivity of the produced anti- *S. haematobium* CP pAb against the purified tegumental antigen along with other parasites (Fasciola, Ancylostoma and Ascaris) were determined by **indirect ELISA**. The immunoglobulin G (IgG) fraction of rabbit anti- *S. haematobium* pAb was purified from the total produced immunoglobulins (pAb) titre using **ammonium sulphate precipitation method** followed by **7% caprylic acid treatment** and finally by using **ion exchange chromatography method (DEAE Sephadex A-50)**.

Test blood samples were withdrawn from New Zealand white rabbit before the injection of each immunizing dose. They were tested for the presence of specific anti-*S. haematobium* antibodies by indirect ELISA. An increasing antibody level started 1 week after the first booster dose. Three days after the 2nd booster dose immune sera gave a high titre against *S. haematobium* CP with OD of 2.97 at 1/250 dilution (**Figure 2.10**)

Since proteins in solutions have been known to form hydrogen bonds with water which, in turn, increase their solubility, **ammonium sulfate precipitation** methods was used to remove these water molecules via competing with them and precipitating the protein molecules after forming more favorable hydrogen bonds.[98] The IgG (The target pAb) was further purified from serum proteins by **caprylic acid** treatment. Protein content was estimated after each purification step using **Bradford assay**. The purity of the produced IgG was identified by 12% SDS-PAGE (1mm) under reducing conditions.

Figure 2.11 shows the OD_{280} profile of the IgG fractions obtained following purification by **DEAE Sephadex A-50 ion exchange column chromatography** by **UV spectrophotometry**. The eluted IgG is represented by a single peak with maximum OD value 2.88 at fraction number 9.

The **total protein content** of crude rabbit serum containing anti- *S. haematobium* CP pAb was found to be 3.1±0.45mg/ml and 2.63±0.89mg/ml after 50% ammonium sulfate precipitation method, while following 7% caprylic acid precipitation method the content

decreased to 2.21±0.19mg/ml. Finally, the protein content of highly purified anti- *S. haematobium* CP IgG pAb subjected to ion exchange chromatography method was 1.99±0.28 mg/ml.

The purity of IgG pAb after each purification step was assayed by **12% SDS-PAGE under reducing conditions**. Analysis of 50% ammonium sulfate- precipitated proteins showed several bands. While the purified IgG pAb after 7% caprylic acid was represented by only two characteristic bands, light (L-) and heavy (H-) chain bands at 31 and 53 kDa, respectively (**Figure 2.12**). General structure of antibody was illustrated in **Figure 2.13**.[63]

Reactivity and specificity of anti- *S. haematobium* CP IgG pAb against *S. haematobium* CP antigen and other parasite antigens (*Fasciola* and *Ascaris*) were assessed by **indirect ELISA**. The produced anti- *S. haematobium* CP IgG pAb gave strong reactivity to *S. haematobium* CP sAg. The mean absorbance readings at 492 nm for *S.haematobium* CP (2.37± 0.12) were significantly higher than that of *Fasciolia* and *Ascaris* infected sera (0.21±011 and 0.27±0.08 for, respectively) as shown in **Table 2.6**.

Section IIB: Preparation and Characterization of PLGA Nanoparticles Surface – Modified With Polyclonal Antibodies

The prime objective of this study was to prepare and evaluate pAb- coated PLGA NPs capable of targeting *Schistosoma* parasites and delivering PZQ near its site of action. As a result of drug targeting, the efficacy of therapy and reduction of side effects associated with the drug can improve.

To achieve targeting of schistosomes, we used polyclonal antibodies (pAb), developed against cysteine proteinases surface antigens (CP sAg) of *Schistosoma* worms specifically *S. haematobium* Egyptian strain, for surface coating of the optimized PZQ- loaded PLGA NPs. Polyclonal antibodies (pAb) were applied instead of monoclonal antibodies (mAb) as they are capable of recognizing several epitopes on the target protein rather than recognizing only single epitope as in case of mAb. Although, mAb are more specific than pAb, sometimes they are not able to precipitate the antigen because the epitope might need to be exposed on the surface of the antigen to be recognized by the antibody. Since some of the epitope might be hidden and it's a single epitope that is recognized by the mAb, the probability of the antibody to recognize the

epitope is lower compared with that of pAb which recognizes several epitopes on the target protein.[118–120]

Two different strategies were applied to attach the amine groups of pAb to the carboxyl groups on PLGA NPs. The first method was depending on the physical non- covalent adsorption; while the second procedure involved the chemical covalent modification of NPs using EDC/ NHS carbodiimide coupling reaction.

In the first method, NPs were incubated with pAb to allow **non-specific adsorption** onto their surface, as reported by Kockbek *et al*.[108] It has been suggested that the adsorption process results generally from the hydrophobic interactions between the non- polar hydrophobic groups of the antibody molecule and the hydrophobic PLGA polymer.[63,109,121] However, in our case, as we prepared the PZQ- loaded PLGA NPs using uncapped acid-terminated PLGA rather than the more commonly used hydrophobic ester-terminated PLGA, several other mechanisms might contribute in the adsorption process as well.

First, hydrophobic interactions might contribute in the adsorption attraction though with a lesser extent in case of acid – terminated PLGA as the exposed non- polar areas on the polymer would be minimized. Wan der Waal forces and hydrogen bonds might be contributors in adsorption as well. Since the adsorption process was allowed to take place at pH 5.5, the slightly acidic pH helps the ionization of surface carboxyl groups. Consequently, the non- covalent adsorption of pAb on the surface of PLGA NPs might be due to electrostatic interactions between the oppositely charged carboxyl and amino groups; however, the carboxylic groups were not being fully activated on the surface of NPs. Even though these interactions are known to be strong enough and even might be stronger than covalent bonds, they could be easily broken with possible changes in the reaction environment like change in pH or addition of water.[63,81,122,123] This might make the adsorption process reversible. Several authors emphasized the importance of competitive displacement of pAb that can take place in the presence of serum components "Plasma proteins". [108] The process of adsorption of peptides to PLGA surface is most favorable when the peptide is uncharged, thus less soluble, and exerts more hydrophobic character that interacts with the hydrophobic surface of polymer.[108]

While in the second method, we assessed **EDC/NHS chemistry for covalent coupling** of PLGA particle surface carboxyl groups and pAb ligand amine groups via amidation reaction. EDC is frequently used for coupling reactions with bioactive molecules; it is a zero length cross linking agent forms stable intermediates with carboxyl groups in the presence of NHS which react with nucleophiles, such as primary amines to form amide bonds.[110,123–127] Furthermore, in the absence of primary amines, the activated intermediates hydrolyze back to the free carboxyl form. Aqueous solubility of EDC and NHS limits exposure of PLGA particles to extreme solvent conditions during activation. **Figure 2.18** is a schematic illustration of the EDC/ NHS carbodiimide crosslinking reaction.

For this reaction, PLGA carboxylic functional groups were first activated through exposure with EDC /NHS in a medium consisting of PBS buffer, pH=5.5; as the carboxyl group should ideally remain mostly in its deprotonated form ($RCOO^-$) so as to act as a nucleophile in the reaction. The activated NPs were then purified and then exposed to pAb solution in PBS, pH =7.4. The conjugated COOH groups were made to react with –NH3+groups present on the pAb molecules to make immuno-NPs. They were then isolated by centrifugation steps and the supernatant was collected for indirect measurement of attached pAb on surface of PLGA NPs. [84]

After purification of immuno-NPs, the amount of coupled pAb on PLGA NPs was quantified using **Bradford assay**. A standard curve constructed using a known BSA concentration range was used for comparison in each experiment as mentioned earlier (**Table 2.4** and **Figure 2.8**). The bioactivity of the pAb on the surface of NPs was assessed by dot blot immuno-assay. In addition, the physical characteristics of PZQ- loaded PLGA NPs were re-evaluated to assess the effect of adsorption and conjugation methods after attachment of pAb on their surfaces.

Table 2.7 summarizes the evaluation results of **pAb- adsorbed PLGA NPs**. The data was expressed as mean ± SD of triplicate experiments. Two parameters were studied: **the incubation time allowed for adsorption** to take place (**Expt.: I-1**) and **the initial amount added of pAb**, µg (**Expt.: I-2**). The amount of PLGA NPs was fixed for all formulae (500 µg). As shown in **Table 2.7**, four ratios of pAb: PLGA (%w/w) were used in **Expt. I-2**, namely: 1:1, 1:25, 1:50 and 1:100. The results presented in **Table 2.7** and **Figure 2.14** demonstrate no significant difference in **the adsorption efficiency** of formulae **A2** (12 hours incubation) and **A4**

(24 hours incubation); though both of them had significantly higher adsorption efficiency if compared with their controls (**A1** and **A3** respectively), *p*-value<0.0001. The adsorption efficiency of pAb to NPs was 37.4± 2.99 and 38.1± 2.05 for **A2** and **A4** respectively with approximately 0.374µg and 0.381 of pAb was adsorbed per mg of NPs.

Regarding the effect of adsorption time on the physical properties of the optimized PZQ-loaded PLGA NPs (**Figure 2.15**), it was noticed that although there was a slight increase in the **size** and **PDI** of pAb- adsorbed PLGA NPs, the difference was non- significant (**Figure 2.15a**). On the contrary, the increase in **zetapotentail** of pAb- adsorbed PLGA NPs was statistically significant than the control (Uncoated PLGA NPs) (**Figure 2.15b**). The charge of **A2** was -11.32±1.99mV which is significantly higher than that of **A1** (-22.01±0.72mV), *p*-value<0.001. Similarly, zetapotential of **A4** was -10.19±2.81mV which is significantly higher than that of **A3** (-21.3±0.91mV), *p*-value<0.001. Yet, there was no difference between **A2** and **A4**. The increase in zetapotential values might be attributed to the attachment of pAb onto the surface of PLGA NPs, masking the free carboxyl groups of PLGA as a result of which the negativity of NPs decreases though not to a great extent that might affect the physical stability of NPs and lead to aggregation of immuno-NPs.[64]

Referring to the results showing the effect of time allowed for adsorption on PZQ **encapsulation efficiency**, it was evident that, although, there was no significant effect on it in both cases of incubation for 12 or 24 hours (**A2** and **A4**) when compared with the controls (**A1** and **A3**). Allowing 24 hours for adsorption lowered the EE% by about more 4% than when incubating the NPs with pAb for 12 hours only (87.02±3.39% instead of 91.89±4.19%, respectively) (**Figure 2.15c**). Based on the above obtained data, 12 hours adsorption time was selected and fixed throughout the next experiment (**I-2**).

Adsorption of various initial pAb amounts had major effect on the amount adsorbed on the surface of PLGA NPs expressed as % weight of adsorbed pAb per mg of PLGA NPs (**Figure 2.14**). Ratio of 1:1 (**A9**) was statistically significant than the other studied ratios (1:25, 1:50 and 1:100 representing formulations **A8**, **A7** and **A6** respectively) in terms of the amount of adsorbed pAb per mg PLGA, *p*-value<0.0001. **A9** showed also significant higher adsorption efficiency than both **A6** and **A7** (64.2±2.87% compared to only 37.4± 5.01 and 36.8±1.99% respectively, *p*-

value<0.0001) but, no significant difference than **A8** in terms of adsorption efficiency (59.5±3.09 %). Accordingly, ratio of 1:1 was chosen as the optimum level along with 12 hours for adsorption time as mentioned earlier, formula number **A9**.

While, **Table 2.8** presents the characterization data of **pAb- conjugated PLGA NPs** via EDC/ NHS covalent conjugation method. Results were represented as mean ± SD of three independent experiments. The effect of **initially added pAb to PLGA NPs ratio** on the conjugation efficiency was studied (**Expt. II-1**). The results illustrated in **Table 2.8** and **Figure 2.16** revealed that formula **C5** prepared using 500µg pAb (1:1 pAb:PLGA, %w/w) showed significantly higher **amount of conjugated pAb per mg of PLGA NPs** than the other three evaluated ratios (1:25 "**C4**", 1:50 "**C3**" and 1:100 "**C2**"), *p*-value< 0.0001(The amount of pAb covalently bound to NPs surface was shown to be approximately 89.31 µg pAb per 1 mg of PLGA NPs.). It also showed higher significant **conjugation efficiency** than **C2** and **C3**, but not statistically higher than **C4**.

By re-evaluating the physical characteristics of the PZQ- loaded PLGA NPs after pAb conjugation (**Figure 2.17**), it was found that there was negligible effect on PZQ **encapsulation efficiency**. This finding was in agreement with that of several previous studies. [84] In contrast, there was significant increase (*p*- value <0.05) in the **zetapotential** of pAb-conjugated PLGA NPs, formulae **C4** and **C5**, in comparison with plain NPs (Control, **C1**) as represented in **Figure 2.17b** (-12.2 ±3.1 mV and -11.98±2.27mv compared with -21.97±1.89mV, respectively); whereas there was non- statistically significant increase in charge of both **C2** and **C3** (-16.7±1.45 and -18.1±2.72mV). This decrease in negative zeta potential is probably due to the involvement of carboxylic functional groups on PLGA NPs in amide bond formation with amino acids in pAb primary structure. This, in turn, support increased association of pAb in the presence of EDC/NHS.[84]

Referring to NPs **size** and **size distribution** results, it was clear that conjugation of pAb on surface of PLGA NPs slightly increased the size and PDI at ratios 1:1 and 1:25 of pAb: PLGA (%/w/w), formulae **C5** and **C4** respectively (222.78±4.56nm and 230.4±4.19nm instead of 202.1 ±3.15nm for plain NPs, **C1**). Interestingly, at ratios 1:50 and 1:100 (Formulae **C3** and **C2**, respectively), there was a huge significant increase in particles' average diameter

(616.98±10.1nm and 637.2±8.12) and PDI (0.398±0.2 and 0.42±0.092). This could be attributed to the presence of active sites on PLGA NPs that interact with the ones on other particles leading to bridging and cross-linking of two or more particles together and in turn the heterogeneous distribution of the NPs population.[84]

On the contrary, at higher initial amounts of pAb added for conjugation, saturation of these activated sites on PLGA NPs was achieved. From these results, it was clear that a ratio of 1:1 (pAb:PLGA) appeared to produce the best conjugation efficiency in terms of the conjugated pAb amount and bioactivity. [64]

The selected optimized pAb- adsorbed PLGA NPs, Formula **A9** and pAb- conjugated PLGA NPs, Formula **C5** (**Table 2.9** and **Figure 2.19**) were evaluated for their targetability. The reactivity of the attached anti- CP pAb was assessed while on the surface of PLGA NPs against *Schistosoma* CP sAg using **dot blot immunoassay** in an indirect manner. [106] In this approach, a known amount of pAb and pAb-PLGA NPs were applied over CP sAg which in turn was spotted and dried onto a NC membrane and then reacted with a peroxidase coupled secondary antibody. A soluble, uncolored substrate was then added which could be converted to an insoluble purple/violet product by the enzyme. Violet color development depended on the amount of enzyme present. Three negative controls were run in parallel (No pAb and two formulae representing uncoated PLGA NPs, Formula A5 and Formula C1). Positive control was also tested using free pAb. Obtained results were represented in **Figure 2.20**.

The intensity of the developed color was directly proportional to the strength of the pAb-sAg interaction. As a result, scores was given to describe the intensity of the color and in turn the bioactivity of pAb ranges from 0 to 3. Score of zero means there was no interaction at all; while scores 1, 2 and 3 (light, moderate and heavy color intensity) indicates weak, medium and strong bioactivity respectively. From **Figure 2.20**, it was clear that the pAb- conjugated NPs reacted strongly with the spotted CP sAg on the membrane. The reaction seems to be stronger than that of the pAb-adsorbed NPs which in turn is weaker than that of free pAb. This might be due to the reversibility of adsorption process as mentioned earlier leading to loss of part of adsorbed antibody during the washing steps of the dot blot immunoassay.

Other PLGA surface functionalization studies reported the loss of bioactivity of pAb after being conjugated to NPs chemically. [108,128,129] This might be due to the involvement of the reactive regions of the pAb in the formation of the amide bond during the conjugation reaction or the conjugation of excess amount of ligand causing steric hindrance of the reactivity towards sAg.[128,129]

It is worth mentioning that several different attempts were made to measure the biological activity of the immuno-NPs in a quantitative manner. Accordingly, standards of purified anti-CP antibody were prepared at serially diluted known concentrations, spotted onto membrane, and treated in an identical manner to that described above. When spot intensity was plotted against antibody concentration the curves were not linear and a standard curve, against which purple color development in samples could be compared, could not be well established (Data not shown). The assay needed to be further optimized and standardized in terms of quantitative estimation of the developed color intensity.

To conclude, in an attempt to formulate immuno-NPs for targeted delivery, pAb coated PZQ- loaded PLGA NPs were developed for targeting *Schistosoma* parasites. On the basis of size, homogeneity of size distribution, zetapotential, encapsulation efficiency, ligand coupling efficiency, amount of attached pAb on the surface of PLGA NPs and the bioactivity of pAb upon attachment to the NPs, the surface functionalization method of the PLGA NPs was hence selected and used for further experimentation. Out of both prepared immuno-NPs, pAb-conjugated immuno-NPs, Formula **C5**, was found to be optimum and can be used further for *in vitro* schistosomicidal studies.[70]

Conclusion

Despite the enormous efforts for control and elimination, schistosomiasis is still an important worldwide health problem in more than 76 countries including Egypt. In fact, this neglected tropical disease exerts substantial medical, economic, and psychosocial impacts on the populations in endemic regions. [11] Unfortunately, schistosomiasis is not assumed to be eliminated in the near future; so, awareness of the various aspects of the disease is still needed in endemic countries.

It is likely that PZQ may remain the anti-schistosomal drug of choice for many years in the future. [17] However, due to its short plasma half-life and rapid metabolism by liver enzymes, the therapeutic potential becomes significantly reduced.[18] Thus, a variety of approaches have been proposed to overcome the limitations faced during its administration. Nanomedicine provides a promising approach for specific drug delivery to avoid the high dose toxicity and the development of resistance against the drug due to massive administration of large doses.

Biocompatible and biodegradable polyester polymers such as PLGA can be formulated into NPs with control over their physical properties. [81] PZQ- loaded PLGA NPs were prepared by single (o/w) emulsion solvent evaporation method and physicochemical characterization of drug loaded NPs system was performed. Small sized NPs (~200nm) with negative zetapotential were obtained that resulted in development of physically stable nano-carriers which could escape the reticuloendothelial system preventing their uptake by macrophages. [84]

Besides, good morphology in terms of the NPs surface, characterization of the physiochemical properties of the encapsulated drug within the NPs was also conducted. DSC and FTIR analysis helped in determining the chemical integrity of PZQ and its interaction with the polymer. Both confirmed that the drug retained its properties even when encapsulated inside NPs. Moreover, a sustained release of PZQ illustrated by the *in vitro* drug-release study indicated proper diffusion of drug from the polymeric PLGA NPs for its effective anti- schistosomal action.

Specific drug delivery can be achieved by targeting that may be either active or passive. [130] In active targeting, different targeting moieties can be used, which are conjugated on to the

surface of NPs. Herein, PLGA NPs surface modified with anti-CP pAb were developed and fully characterized. Non- covalent adsorption and direct covalent coupling of antibodies to surface of PLGA NPs were compared as two different surface functionalization methods to achieve active – targeted and specific drug carrier systems. The pAb- chemically conjugated to PLGA NPs, in which the amine groups of the anti-CP pAb were made to covalently couple with the activated carboxylic groups present at the surface of PLGA NPs in the presence of EDC/NHS by forming an amide linkage, were selected for further *in vitro* schistosomicidal studies.

The newly developed NPs had an acceptable size and zetapotential and showed promising results in both adult and juvenile *S. haematobium* cultures (Data not shown). It has been shown that the anti- schistosomal activity of PZQ increased after conjugating a targeting ligand, anti-CP pAb, onto the PLGA NPs surface. Specific targeting and higher uptake of the immuno-NPs by schistosome parasites resulted in enhanced anti- schistosomal effect of the encapsulated drug, PZQ.

It seemed that surface modification of PLGA NPs with anti- CP may represent a promising approach for active targeting of schistosomiasis. We even can postulate that a similar approach may be used to develop drug loaded immuno-NPs for treatment of different human diseases mainly in the field of neglected parasitic diseases in near future.[48]

Such a nano- carrier system for PZQ delivery is multifunctional, which have several benefits. It reduces the side effects of the formulated drug, promotes synergistic therapeutic effects between the bioactive ligand (anti- schistosome pAb) and entrapped drug, and achieves targeted delivery of therapeutic drug. Thus, the developed drug delivery system (DDS) was able to provide an efficient and targeted delivery of PZQ for schistosomiasis treatment.

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Appendixes

Appendix I: Materials*

**All used Solvents were analytical grade and were used without further purification. All aqueous solutions were prepared with twice distilled and degassed water, deionized and filtered with Millipore Elix 3 System.*

- DL-Lactide/Glycolide copolymer (PLGA), Lactide/Glycolide ratio 75:25 with intrinsic viscosity 0.2 dl/g, PURASORB PDLG 7502A, Purac Biomaterials, Netherlands
- DL-Lactide/Glycolide copolymer (PLGA), Lactide/Glycolide ratio 50:50 with intrinsic viscosity 0.4 dl/g, PURASORB PDLG 5004A, Purac Biomaterials, Netherlands
- Dichloro methane (DCM), analytical grade, Sigma- Aldrich, USA
- Ethyl Acetate (EA), puriss, Sigma-Aldrich Co., USA
- Acetone, Sigma-Aldrich Co., USA
- Poly vinyl alcohol (PVA), 87-90% hydrolyzed, M W 30,000-70,000, Sigma-Aldrich, USA
- Poloxamer 407, Sigma-Aldrich Co., USA
- Poloxamer 188, Sigma-Aldrich Co., USA
- Tween 80, Sigma-Aldrich Co., USA
- Praziquantel, Sigma-Aldrich Co., USA
- L- Leucine, Fluka, Switzerland
- Sucrose, Fluka, Switzerland
- N-hydroxysuccinamide (NHS), Sigma-Aldrich Co., USA
- 1-Ethyl-3-(dimethylaminopropyl) carbodiimide hydrochlo- ride (EDC), Sigma-Aldrich Co., USA
- Tris-HCl, Bio-Rad , Richmond, CA, USA
- Sephadex A-50 powder, Amersham Bioscience, Uppsala, Sweden
- Sephacryl S-100 HR, Amersham Bioscience, Uppsala, Sweden
- 30x2.5 cm Column, Biorad

- Coomassie brilliant blue G-250 dye Bradford Dye, Bio-Rad, Richmond, CA, USA
- Nitrocellulose membrane, Millipore, Billerica, MA, USA
- Rabbit-anti-mouse secondary antibody, Imgenex Corporation, San Diego, USA
- TMB Liquid Substrate System – Sigma-Aldrich Company Ltd, Gillingham, UK
- Sodium acetate buffer pH 4.8, Sigma Aldrich, USA
- Caprylic acid (Octanoic acid) best grade, Sigma Aldrich, USA
- O-phenylene diamine dihydrochloride (OPD), Sigma Aldrich, USA
- Microtiter plates, Costar, Corporate Headquarters, Cambridge, MA, USA
- Roswell Park Memorial Institute (RPMI-1640) with l-glutamine, Life Biosciences
- Fetal Bovine Serum (FBS), Sigma Aldrich, USA
- 100 IU/ml Penicillin, 100 µg/ml Streptomycin, Life Biosciences
- Gentamycin, Life Biosciences
- Sterile, flat-bottom, 24-well plates, Corning, NY, USA

Appendix II: Equipment

- Ultraturrax T25 laboratory Emulsifier /Homogenizer, Ika, Staufen, Germany
- Qsonica Q700 Probe Sonicator, Qsonica, LLC., Newtown, USA
- Vortex, Fisher Scientific, Malaysia
- Magnetic Stirrer, Fisher Scientific, Malaysia.
- Ultra-Centrifugation with cooling unit, Sorvall RC 6 plus Centrifuge
- Lyophilizer, Labconco Corporation, Kansas City, MO
- Rotary evaporator, BUCHI R114, BÜCHI Labortechnik AG, Switzerland
- Particle size analyzer (Zetasizernano ZS), Malvern Zetasizer, Malvern Instruments Ltd., Malvern, UK
- Digital Balance, SI 234, Denver instrument, USA
- ELISA plate Reader, Synergy HT, BioTek Instruments Inc., Winooski, Vermont
- UV Spectrophotometer (Perkin-Elmer Lambda 1A)
- Cooling Centrifuge, Eppendorf Centrifuge 5415
- Epson Perfection 1640SU scanner, Tokyo, Japan
- Perfusion Pump MasterFlex pump, Cole-Parmer, Vernon Hills, Illinois
- Light microscope, Olympus Inverted Microscope Model IX70; Olympus, Tokyo, Japan
- Ziess Scanning Electron Microscope model
- Vecco Atomic Force Microscope

Appendix III: Tables

Table 1.1: Summary of the Studied Factors Affecting the Properties of PLGA NPs in the Preliminary Study (Study I): Various Formulae and their Composition.

Expt. #	Studied Factor	F#	Variables*	Experimental Conditions
I -1	Organic Solvent Type (Preparation Method)	P1	DCM (ESE)	Homogenization only at 22,000 rpm for 20 min, Rotavap, Ultracentrifugation, No leucine in the external aqueous phase
		P2**	EA (ESD)	Homogenization at ascending speed (5000, 10000 and 20000 rpm - 3 min each). Then, homogenization only at 22,000 rpm for 3 min, Rotavap, Ultracentrifugation, No leucine in the external aqueous phase
		P3**	Acetone (Nanopptn)	Stirring at 800rpm for 24hrs at RT, Ultracentrifugation, No leucine in the external aqueous phase
I-2	Evaporation Technique	P4	Rotavap	Rotavap at 45^{o}C for 20 min
		P5	Stirring Under Atmospheric Conditions	Stirring at RT overnight
I-3	Evaporation time (Hours)	P6	2	Evaporation for 2 hours
		P7	4	Evaporation for 4 hours
		P8	6	Evaporation for 6 hours
		P9	12	Evaporation for 12 hours
I-4	Purification Technique	P10	Ultracentrifugation	Ultracentrifugation (20000 rpm, 4^{o}C, 20 min, washing twice)
		P11	Centrifugation	Centrifugation (11000 rpm, 4^{o}C, 30 min, washing twice)
I-5	Addition of Antiaggregant in Aqueous Phase	P12	Absence	No antiaggregant in the external aqueous phase
		P13	Presence	0.01% leucine as antiaggregant in the external aqueous phase
I-6	Emulsification Technique	P14	Homogenization	Homogenization only at 22,000 rpm for 20 min
		P15	Sonication	Continueous Sonication using probe sonicator only at 100% amplitude for 20 min
		P16	Homogenization and Sonication	Homogenization at ascending speed (5000, 10000 and 20000 rpm - 3 min each). Then, probe sonication (pulse on 30 sec and pulse off 5 sec) for 20 min

Expt. #	Studied Factor	F#	Variables*	Experimental Conditions
I-7	Sonication Time	**P17**	20 min	Homogenization at ascending speed (5000, 10000 and 20000 rpm - 3 min each). Then, probe sonication (pulse on 30 sec and pulse off 5 sec) for 20 min
		P18	10 min	Homogenization at ascending speed (5000, 10000 and 20000 rpm - 3 min each). Then, probe sonication (pulse on 30 sec and pulse off 5 sec) for 10 min
		P19	5 min	Homogenization at ascending speed (5000, 10000 and 20000 rpm - 3 min each). Then, probe sonication (pulse on 30 sec and pulse off 5 sec) for 5 min
I-8	Effect of Lyophilization	**P20**	Not Lyophilized	No lyophilization
		P21	Lyophilized	Freeze drying at -80^{o}C and <10 mm mercury pressure for 48 hours after addition of 0.2% sucrose as cryoprotectant

* Fixed levels of other factors throughout all experiments of this study are: PLGA 75:25 5% w/v, Organic Solvent 5% v/v (DCM in all formulae except **P2** and **P3**), PVA 1% w/v, D:P (1:6), Initial Drug Weight (50mg)

** For **P2** and **P3** refer to sections I.A.1.2 and I.A.1.3 respectively.

Table 1.2: Summary of the Studied Factors Affecting the Properties of PLGA NPs in the Screening Phase (Study II): Various Formulae and their Composition.

Expt. Number	Screened Variable	F#	Levels*
II-1	Polymer Concentration	**S1**	1%
		S2	2.5%
		S3	5%
II-2	Organic Solvent %	**S4**	1%
		S5	2.5%
		S6	5%
II-3	SAA Type	**S7**	Poloxamer 407
		S8	TW 80
		S9	Poloxamer 188
		S10	PVA

* Fixed levels of other factors throughout all experiments of this study are: PLGA 75:25 , DCM, SAA 1% w/v, **Non-medicated**

Table 1.3: Summary of the Studied Factors Affecting the Properties of PLGA NPs in the Validation Phase I (Study III): Various Formulae and their Composition.

Expt. Number	Validation Using (Polymer Type/ Characteristics)	F#	Formula Type
III-1	PLGA 75:25 (M.wt. 17,000 Da "0.2 dl/g inherent viscosity", Uncapped)	**VI1**	Blank
		VI2	PZQ - Loaded
III-2	PLGA 50:50 (M.wt. 44,000 Da "0.4 dl/g inherent viscosity", Uncapped)	**VI3**	Blank
		VI4	PZQ - Loaded

Fixed levels of other factors throughout all experiments of this study are: PLGA 5% w/v, DCM 5% v/v, PVA 1% w/v, D:P (1:6)

Table 1.4: Summary of the Studied Factors Affecting the Properties of PLGA NPs in the Optimization Phase (Study IV): Various Formulae and their Composition.

Expt. Number	Optimized Variable	F#	Levels
IV -1	SAA %	**O1**	0.5 %
		O2	1%
		O3	2%
		O4	4%
IV -2	Addition of Salt to External/Aqueous Phase (Salt Type)	**O5**	NaCl
		O6	$(NH_4)_2 SO_4$
IV -3	Addition of Salt to External/Aqueous Phase (Salt %)	**O7**	5
		O8	10
		O9	20
IV-4	D:P	**O10**	1:1
		O11	1:2
		O12	1:6
		O13	1:8
		O14	1:10

Fixed levels of other factors throughout all experiments of this study are: PLGA 50:50 5% w/v, DCM 5% v/v, PVA

Table 1.5: Summary of the Studied Factors Affecting the Properties of PLGA NPs in the Validation Phase II (Study V): Various Formulae and their Composition.

Expt. Number	Formulae Prepared using selected optimum levels of the investigated parameters	F#	Formula Type
V-1	1:1 (Effect of Initial Drug Weight)	**VII1**	Blank
		VII2	1:1 (50mg)
		VII3	Blank
		VII4	1:1 (100mg)
V-2	1:2 (Effect of Initial Drug Weight)	**VII5**	Blank
		VII6	1:2 (50mg)
		VII7	Blank
		VII8	1:2 (100mg)

Fixed levels of other factors throughout all experiments of this study are: PLGA 50:50 5% w/v, DCM 5% v/v, PVA 0.5% w/v, $(NH_4)_2 SO_4$ 5% w/v

Table 1.6: Concentrations of Praziquantel Used for Establishment of Calibration Curve Using HPLC Metthod.

Concentration (µg/ml)	AUC average	±SD
2	12.741	0.685
4	26.452	0.1
7	43.201	0.456
10	63.244	0.1
13	83.174	0.277
15	94.585	1.75

Table 1.7: Different Characteristics of PZQ-loaded PLGA NPs Formulations Prepared in the Preliminary Study (Study I). Results were represented as Mean±SD of three independent experiments.

Phase	Expt #	F#	Variable	Particle Size nm		PDI		Zeta-potential mV		EE%		DL%	
				Mean	(±SD)	Mean	(±SD)	Mean	(±SD)	Mean	(±SD)	Mean	(±SD)
Study I: Preliminary Phase	I -1	P1	DCM (ESE)	281.3	3.98	0.61	0.093	-28.5	4.23	4.2	0.85	0.6	0.85
		P2	EA (ESD)	341.83	9.4	0.71	0.241	-26.93	2.98	4.15	0.99	0.592	0.99
		P3	Acetone (Nanopptn)	96.71	4.8	0.59	0.19	-26.37	3.67	2.7	1.13	0.385	1.13
	I-2	P4	Rotavap	281.3	3.98	0.61	0.23	-28.5	4.23	4.2	0.85	0.6	0.85
		P5	Stirring Under Atmospheric Conditions	290.8	2.82	0.57	0.19	-27.99	2.91	2.13	1.45	0.304	1.45
	I-3	P6	2	249.6	3.33	0.52	0.21	-26.1	1.31	4.27	2.46	0.61	2.46
		P7	4	265.8	4.08	0.42	0.156	-27.27	2.56	3.75	1.21	0.53	1.21
		P8	6	272	3.83	0.48	0.27	-27.27	1.89	3.23	2.95	0.46	2.95
		P9	12	290.8	2.82	0.57	0.19	-27.99	2.91	2.13	1.45	0.304	1.45
	I-4	P10	Ultracentrifugation	249.6	3.33	0.52	0.21	-26.1	1.31	4.27	2.46	0.61	2.46
		P11	Centrifugation	241.1	2.67	0.38	0.13	-25.2	1.78	4.95	2.11	0.707	2.11
	I-5	P12	Absence	241.1	2.67	0.38	0.13	-25.2	1.78	4.95	2.11	0.707	2.11
		P13	Presence	239.2	1.87	0.29	0.11	-24.9	1.33	4.78	1.9	0.682	1.9
	I-6	P14	Homogenization	239.2	1.87	0.29	0.11	-24.9	1.33	4.78	1.9	0.682	1.9
		P15	Sonication	230.74	2.11	0.583	0.155	-25.11	2.9	4.39	3.9	0.627	3.9
		P16	Homogenization and Sonication	222.97	2.09	0.07	0.09	-26.08	4.1	4.31	4.5	0.615	4.5
	I-7	P17	20 min	222.97	2.09	0.07	0.09	-26.08	4.1	4.31	4.5	0.615	4.5
		P18	10 min	246.63	3.86	0.321	0.095	-26.73	5.21	5.28	3.67	0.754	3.67
		P19	5 min	280.3	3.98	0.509	0.085	-25.91	5.17	4.97	2.71	0.71	2.71
	I-8	P20	Not Lyophilized	222.97	2.09	0.07	0.09	-26.08	4.1	4.31	4.5	0.615	4.5
		P21	Lyophilized	227.23	4.8	0.12	0.04	-27.84	3.19	4.07	3.73	0.581	3.73

Table 1.8: Different Characteristics of PZQ-loaded PLGA NPs Formulations Prepared in the Screening Phase (Study II). Results were represented as Mean±SD of three independent experiments.

Phase	Expt #	F#	Variables	Particle Size nm		PDI		Zetapotential mV	
				Mean	(±SD)	Mean	(±SD)	Mean	(±SD)
Study II: Screening Phase	II-1	S1	1% PLGA	214.33	4.09	0.38	0.1	-24.65	2.8
		S2	2.5% PLGA	218.17	7.57	0.28	0.08	-26.11	1.1
		S3	5% PLGA	225.77	0.92	0.09	0.02	-29.39	3.9
	II-2	S4	1% DCM	290.3	3.75	0.49	0.1	-23.67	3.1
		S5	2.5% DCM	262.7	2.71	0.33	0.09	-25.98	2.8
		S6	5% DCM	225.77	0.92	0.09	0.02	-29.39	3.9
	II-3	S7	Poloxamer 407	448.22	8.6	0.53	0.1	-27.8	2.8
		S8	TW 80	545.97	7.3	0.89	0.08	-28.1	4.9
		S9	Poloxamer 188	361.57	6.95	0.47	0.08	-27.3	3.4
		S10	PVA	225.77	0.92	0.09	0.02	-29.39	3.9

Table 1.9: Different Characteristics of PZQ-loaded PLGA NPs Formulations Prepared in the Validation Phase I (Study III). Results were represented as Mean±SD of three independent experiments.

Phase	Expt #	F#	Variable	Particle Size nm		PDI		Zetapotential mV		EE%		DL%	
				Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Study III: Validation Phase I	III-1	VI1	PLGA 75:25 (M.wt. 17,000 Da "0.2 dl/g inherent viscosity", Uncapped)	225.77	0.92	0.09	0.02	-29.39	3.9	NA	NA	NA	NA
		VI2		222.97	2.09	0.07	0.09	-26.08	4.1	4.31	4.5	0.615	4.5
	III-2	VI3	PLGA 50:50 (M.wt. 44,000 Da "0.4 dl/g inherent viscosity", Uncapped)	206.6	0.72	0.07	0.03	-25.9	0.92	NA	NA	NA	NA
		VI4		214.73	1.43	0.05	0.02	-24.5	0.62	8.08	1.1	1.15	1.1

Table 1.10: Different Characteristics of PZQ-loaded PLGA NPs Formulations Prepared in the Optimization Phase (Study IV). Results were represented as Mean±SD of three independent experiments.

Phase	Expt #	F#	Variable	Particle Size nm		PDI		Zetapotential mV		EE%		DL%	
				Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Study IV: Optimization Phase	IV -1	O1	0.5 % PVA	229.3	2.46	0.04	0.03	-24.83	0.10	10.39	1.65	1.47	1.65
		O2	1% PVA	214.73	1.43	0.05	0.02	-24.5	0.62	8.08	1.1	1.15	1.1
		O3	2% PVA	213	2.5	0.07	0.03	-20.6	0.15	7.58	1.21	1.082	1,21
		O4	4% PVA	188.57	5.63	0.15	0.08	-18.3	0.89	4.15	3.09	0.592	3.09
	IV -2	O5	NaCl	240.1	4.95	0.18	0.02	-24.57	0.32	27.22	1.17	3.95	1.17
		O6	$(NH_4)_2 SO_4$	232.3	3.18	0.19	0.01	-21.9	0.4	26.33	1	3.81	1
	IV -3	O7	5% $(NH_4)_2 SO_4$	232.3	3.18	0.19	0.01	-21.9	0.4	26.33	1	3.81	1
		O8	10% $(NH_4)_2 SO_4$	610.1	6.56	0.58	0.07	-22.23	1.12	65.38	1.58	9.29	1.58
		O9	20% $(NH_4)_2 SO_4$	608.2	4.7	0.58	0.12	-21.93	1.86	99.9	5	14.27	5
	IV-4	O10	D:P (1:1)	193.6	1.89	0.09	0.01	-21.78	0.89	37.9	2.1	12.63	2.1
		O11	1:2	200.3	2.75	0.18	0.02	-24.21	1.23	21.65	2.1	7.216	2.1
		O12	1:6	214.73	1.43	0.05	0.02	-24.5	0.62	8.08	1.1	1.15	1.1
		O13	1:8	236.8	4.93	0.11	0.03	-26.27	0.5	6.95	2.76	0.772	2.76
		O14	1:10	237.2	2.66	0.15	0.01	-28.5	1.17	3.65	5.43	0.331	5.43

Table 1.11: Different Characteristics of PZQ-loaded PLGA NPs Formulations Prepared in the Validation Phase II (Study V). Results were represented as Mean±SD of three independent experiments.

Phase	Expt #	F#	Variable	Particle Size nm		PDI		Zetapotential mV		EE%		DL%	
				Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Study V: Validation Phase II	V-1	VII1	1:1 (Effect of Initial Drug Weight)	185.9	4.04	0.1	0.01	-24.57	0.32	NA	NA	NA	NA
		VII2		200.1	4.68	0.19	0.02	-21.4	2.10	77.16	2.05	38.59	2.05
		VII3		187.43	2.78	0.05	0.01	-23.98	2.98	NA	NA	NA	NA
		VII4		200.8	4.02	0.13	0.08	-21.03	3.08	94.19	3.46	47.095	3.46
	V-2	VII5	1:2 (Effect of Initial Drug Weight)	192.8	4.09	0.12	0.01	-27.5	1.73	NA	NA	NA	NA
		VII6		204.8	3.89	0.09	0.05	-25.9	2.99	45.77	1.98	15.255	1.98
		VII7		201.4	3.46	0.15	0.03	-28.1	2.34	NA	NA	NA	NA
		VII8		205	4.81	0.14	0.01	-25.4	1.61	66.95	1.63	22.52	1.63

Table 1.12: Release Profile of PZQ (Cumulative % Release Over Time) from the optimized Formula VII4 in Comparison with Pure PZQ Powder. Data was present as Mean±SD of triplicate experiment.

Time		PZQ Cumulative Release			
		Control	±SD	**VII4**	±SD
Hours	**0.25**	**10.86**	3.15	**10.64**	4.08
	0.5	**13.14**	4.21	**10.57**	3.79
	0.75	**21.84**	4.97	**14.77**	4.32
	1	**28.48**	6.15	**16.68**	2.98
	2	**36.32**	3.85	**24.42**	2.67
	3	**44.78**	1.87	**32.61**	3.05
	6	**62.49**	3.76	**47.99**	4.19
	9	**67.54**	2.97	**49.9**	5.01
	12	**77.96**	3.11	**54.99**	4.29
Days	**1**	**93.77**	2.02	**54.21**	2.88
	2	**94.97**	4.68	**63.44**	3.43
	3	**95.85**	3.91	**63.77**	1.93
	7	**91.63**	1.76	**63.67**	4.09
	14	**91.43**	2.67	**62.46**	2.74
	21	**93.89**	4.05	**67.98**	3.99
	28	**94.67**	2.99	**72.69**	1.89
	35	**94.21**	1.67	**79.82**	2.45

Table 2.1: Optimum PZQ - loaded PLGA NPs prepared by the optimized ESE method.

F#	Internal Organic Phase					External Aqueous Phase					Particle Size nm		PDI		Zetapotential mV		EE%		DL%	
	Polymer % w/v	Organic Solvent Type	Organic Solvent % v/v	D:P	Initial Drug Weight mg	Aqueous Solvent % v/v	SAA Type	SAA % w/v	Salt Type	Salt % w/v	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
VII3	5%	DCM	5%	NA	NA	95%	PVA	0.5%	$(NH_4)_2SO_4$	5%	187.43	2.78	0.05	0.01	-23.98	2.98	NA	NA	NA	NA
VII4	5%	DCM	5%	1:1	100	95%	PVA	0.5%	$(NH_4)_2SO_4$	5%	200.8	4.02	0.13	0.08	-21.03	3.08	94.19	3.46	47.095	3.46

Table 2.2: Different Variables of Two Studied Parameters for Preparation of Immuno- NPs Formulations surface-functionalized via Physical Adsorption of pAb.

Coating Method	Expt. #	Parameter	F#	Formula Type	Variables
Physical Adsorption	**I-1**	Time of Adsorption (5µg pAb)	**A1**	Control	12 Hours
			A2	pAb*	
			A3	Control	24 Hours
			A4	pAb*	
	I-2	Amount of Antibody (pAb : PLGA)	**A5**	Control	No pAb
			A6	pAb	5 µg **(1:100)**
			A7	pAb	10 µg **(1:50)**
			A8	pAb	20 µg **(1:25)**
			A9	pAb	500 µg **(1:1)**

Table 2.3: Immuno- NPs Formulations surface-functionalized through Chemical Conjugation of pAb via EDC/NHS Carbodiimide Chemistry.

Coating Method	Expt. #	Parameter	F#	Formula Type	Variables
Chemical Conjugation	**II-1**	Amount of Antibody (pAb : PLGA)	**C1**	Control	No pAb
			C2	pAb	5 µg **(1:100)**
			C3	pAb	10 µg **(1:50)**
			C4	pAb	20 µg **(1:25)**
			C5	pAb	500 µg **(1:1)**

Table 2.4: Concentrations Used for BSA Standard Curve Construction and the Measured Absorbance at 595 Using Bradford Protein Quantification Assay. Results were represented as Mean±SD of triplicate measurements.

Concentration (µg/ml)	Absorbance at 595nm		K
	Mean	±SD	
2000	1.321	0.110	1513.546
1500	1.159	0.041	1294.48
1000	0.782	0.003	1278.881
750	0.639	0.015	1173.219
500	0.484	0.016	1032.631
250	0.236	0.014	1061.121
125	0.121	0.018	1029.937
25	0.023	0.024	1101.322
25	0.7022	0.025322	35.60239
20	0.5985	0.015938	33.41688
10	0.281567	0.007047	35.51557
5	0.093067	0.005558	53.72493
2.5	0.106767	0.020951	23.41555

Table 2.5: Reactivity of Purified Target *S. haematobium* CP antigen Determined by Indirect ELISA. Results were expressed as Mean±SD of 3 measurements.

Serum Samples	Absorbance at 492 nm "Mean (± SD)"
Schistosomiasis (S. haematobium)	1.41 (0.09)
Fascioliasis	0.12 (0.076)
Ascariasis	0.18 (0.092)

Table 2.6: Reactivity and Specificity of Anti-*S.haematobium* CP IgG pAb Against Different Parasitic Antigens by Indirect ELISA. Results were expressed as Mean±SD of 3 measurements.

Parasitic Antigen	Absorbance at 492 nm "Mean (± SD)"
Schistosomiasis (S. haematobium)	2.37 (0.12)
Fascioliasis	0.21 (0.11)
Ascariasis	0.27 (0.08)

Table 2.7: Surface coating of optimum PZQ - loaded PLGA NPs by physical adsorption of pAb: Various Immuno-NPs Characteristics. Results were expressed as Mean±SD of triplicates.

Coating Method	Expt. #	Parameter	F#	Formula Type	Variables	pAb Adsorption (%w/w of PLGA weight)		Adsorption Efficiency %		Particle Size nm		PDI		Zetapotential mV		EE%	
						Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Physical Adsorption	**I-1**	Time of Adsorption (5µg pAb)	**A1**	Control	12 Hours	0	0	0	0	198.9	3.04	0.09	0.026	-22.01	0.72	91.21	4.01
			A2	pAb*		0.374	0.283	37.4	2.99	207.2	3.87	0.19	0.02	-11.32	1.99	91.89	4.19
			A3	Control	24 Hours	0	0	0	0	199.1	4.56	0.16	0.051	-21.3	0.91	86.82	4.48
			A4	pAb*		0.381	0.176	38.1	2.05	209.08	3.49	0.18	0.1	-10.19	2.81	87.02	3.39
	I-2	Amount of Antibody (pAb : PLGA)	**A5**	Control	No pAb	0	0	0	0	198.9	4.21	0.09	0.082	-22.01	2.47	91.21	3.1
			A6	pAb	5 µg (1:100)	0.374	0.412	37.4	5.01	207.2	4.98	0.19	0.065	-11.32	2.94	91.89	4.2
			A7	pAb	10 µg (1:50)	0.736	0.409	36.8	1.99	211.76	3.02	0.18	0.068	-11.12	2.1	90.91	3.31
			A8	pAb	20 µg (1:25)	2.38	0.367	59.5	3.09	210.21	3.71	0.18	0.09	-11.7	3.01	91.32	3.19
			A9	pAb	500 µg (1:1)	64.2	3.91	64.2	2.87	218.23	3.03	0.15	0.21	-10.34	2.57	90.89	4.19

Table 2.8: Surface coating of optimum PZQ - loaded PLGA NPs by Chemical Conjugation of pAb via Carbodiimide Reaction: Various Immuno-NPs Characteristics. Results were expressed as Mean±SD of triplicates.

Coating Method	Expt. #	Parameter	F#	Formula Type	Variables	pAb Conjugation (%w/w of PLGA weight)		Conjugation Efficiency %		Particle Size nm		PDI		Zetapotential mV		EE%	
						Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Chemical Conjugation	**II-1**	Amount of Antibody (pAb : PLGA)	**C1**	Control	No pAb	0	0	0	0	202.1	3.15	0.11	0.089	-21.97	1.89	88.96	4.89
			C2	pAb	5 µg (1:100)	0.784	0.21	78.4	1.64	637.2	8.12	0.42	0.092	-16.7	1.45	88.5	4.19
			C3	pAb	10 µg (1:50)	1.59	0.278	79.8	2.39	616.98	10.1	0.398	0.2	-18.1	2.72	88.71	5.01
			C4	pAb	20 µg (1:25)	3.26	1.98	81.65	2.29	230.4	4.19	0.19	0.063	-12.2	3.1	88.67	2.67
			C5	pAb	500 µg (1:1)	89.31	3.1	89.31	1.78	222.78	4.56	0.16	0.046	-11.98	2.27	89.43	3.92

Table 2.9: Summary of Various Physical Properties of the Optimized Uncoated PZQ- loaded PLGA NPs and pAb- Coated NPs. Data were expressed as Mean±SD (n=3).

	PZQ- loaded PLGA NPs VII4		**pAb- coated PLGA NPs (Adsorption) A9**		**pAb- coated PLGA NPs (Conjugation) C5**	
	Mean	**±SD**	**Mean**	**±SD**	**Mean**	**±SD**
Particle Size nm	200.8	4.02	218.23	3.03	222.78	4.56
PDI	0.13	0.08	0.15	0.21	0.16	0.046
Zetapotential mV	-21.03	3.08	-10.34	2.57	-11.98	2.27
EE%	94.19	3.46	90.89	4.19	89.43	3.92

Appendix IV: Figures

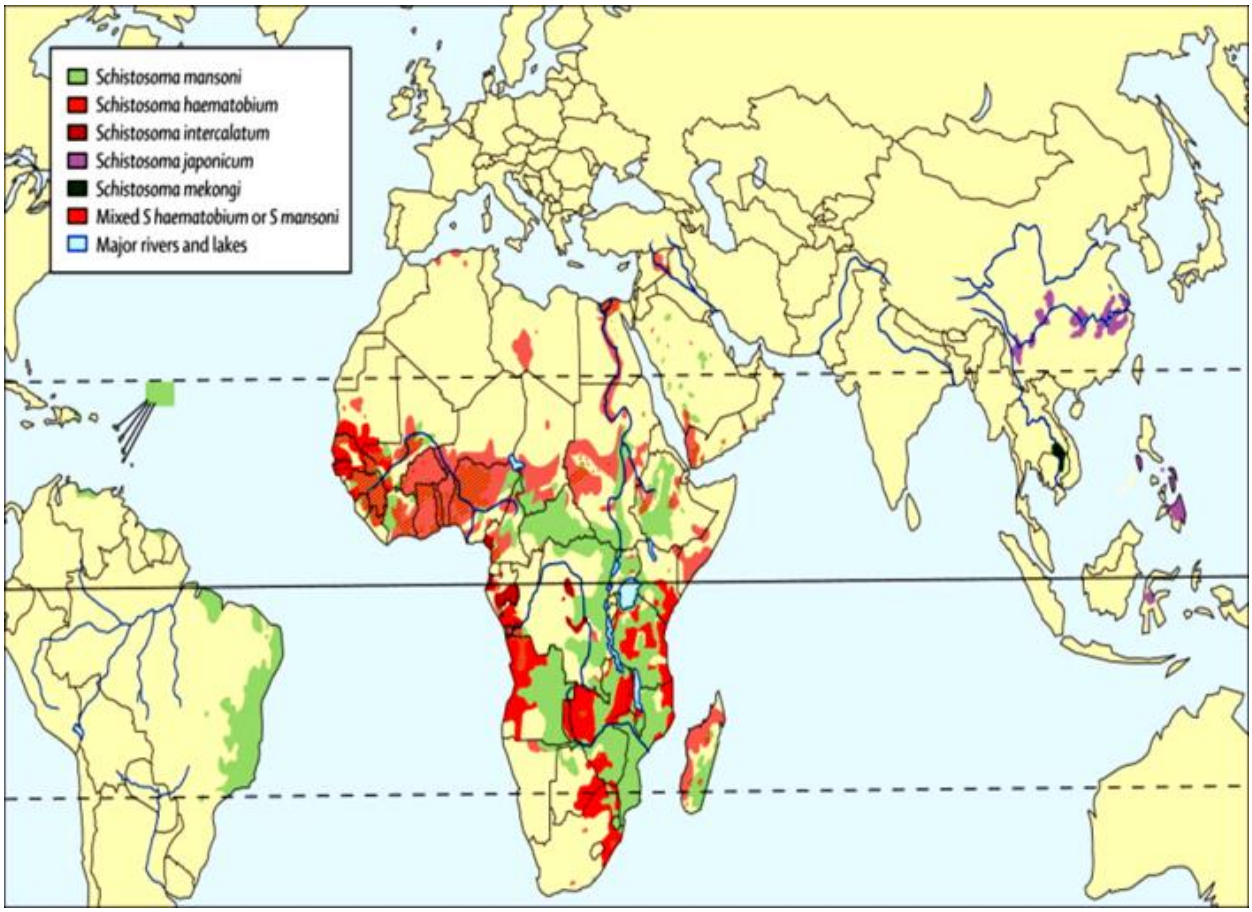

Figure 1: Global distribution of schistosomiasis.[9] (Reproduced with Permission "Appendix V")

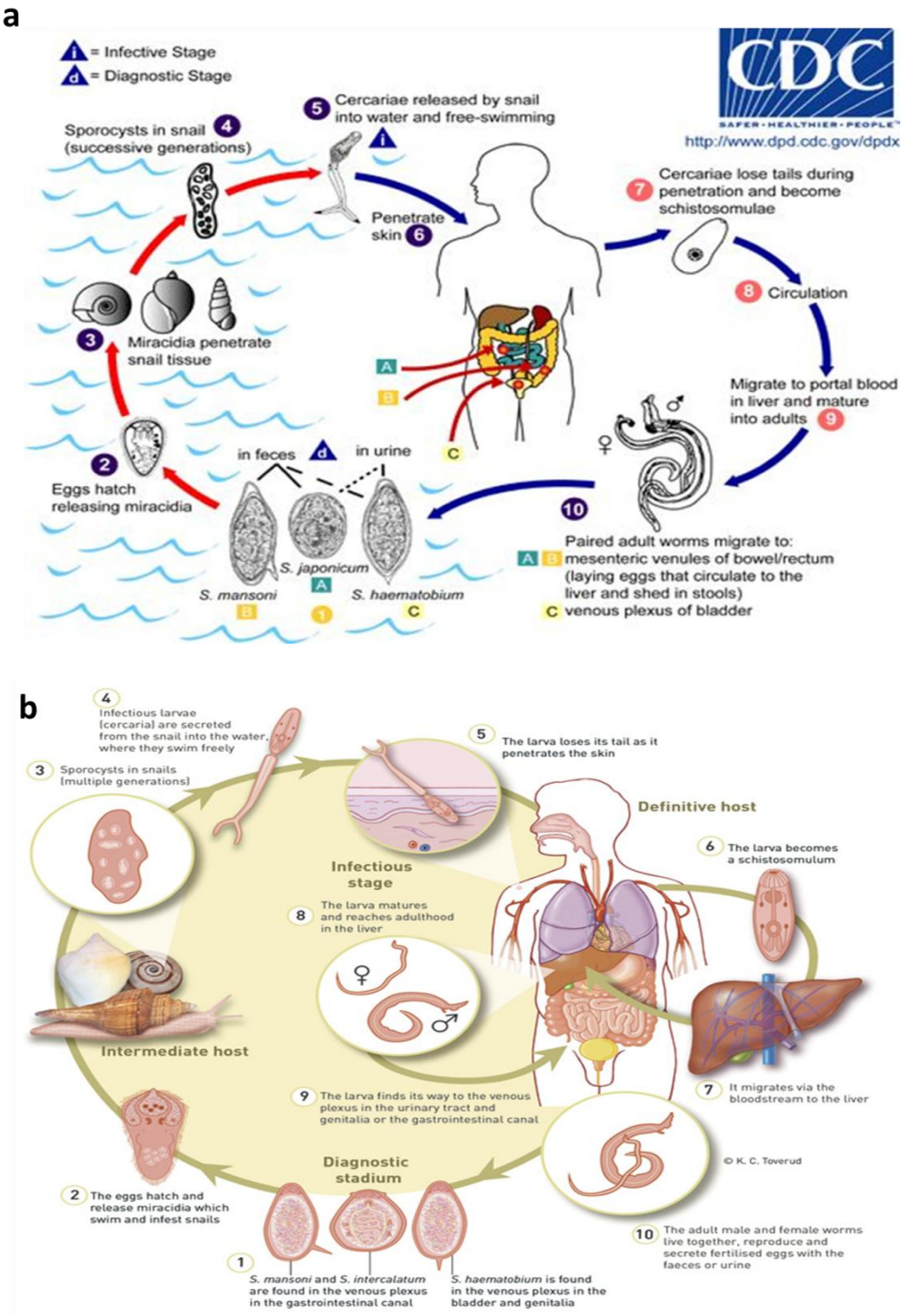

Figure 2: Schistosome Parasite Life Cycle. [131, 132]

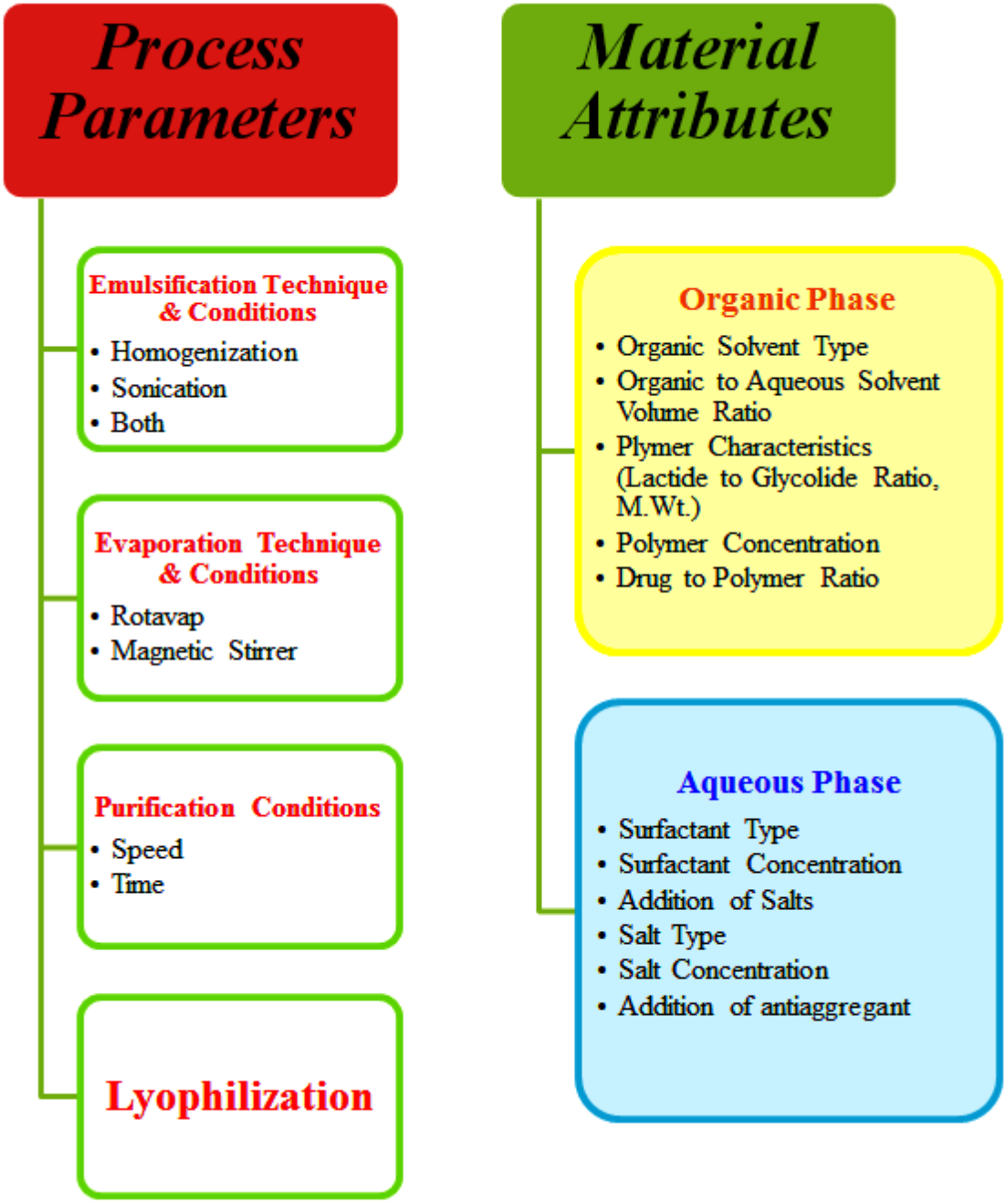

Figure 1.1: Various Formulation Parameters Studied for Controlling the Properties PZQ-loaded PLGA Nanoparticles.

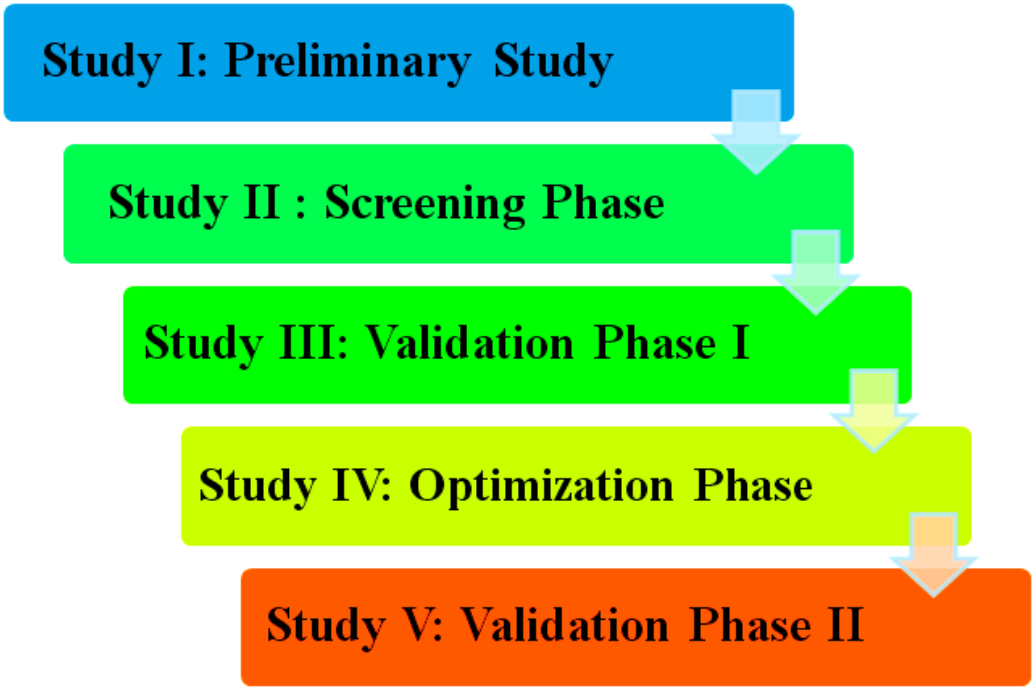

Figure 1.2: Five Main Studies in "*Chapter One*" for Optimization of the Formulation and Experimental Parameters for the Preparation of PLGA NPs.

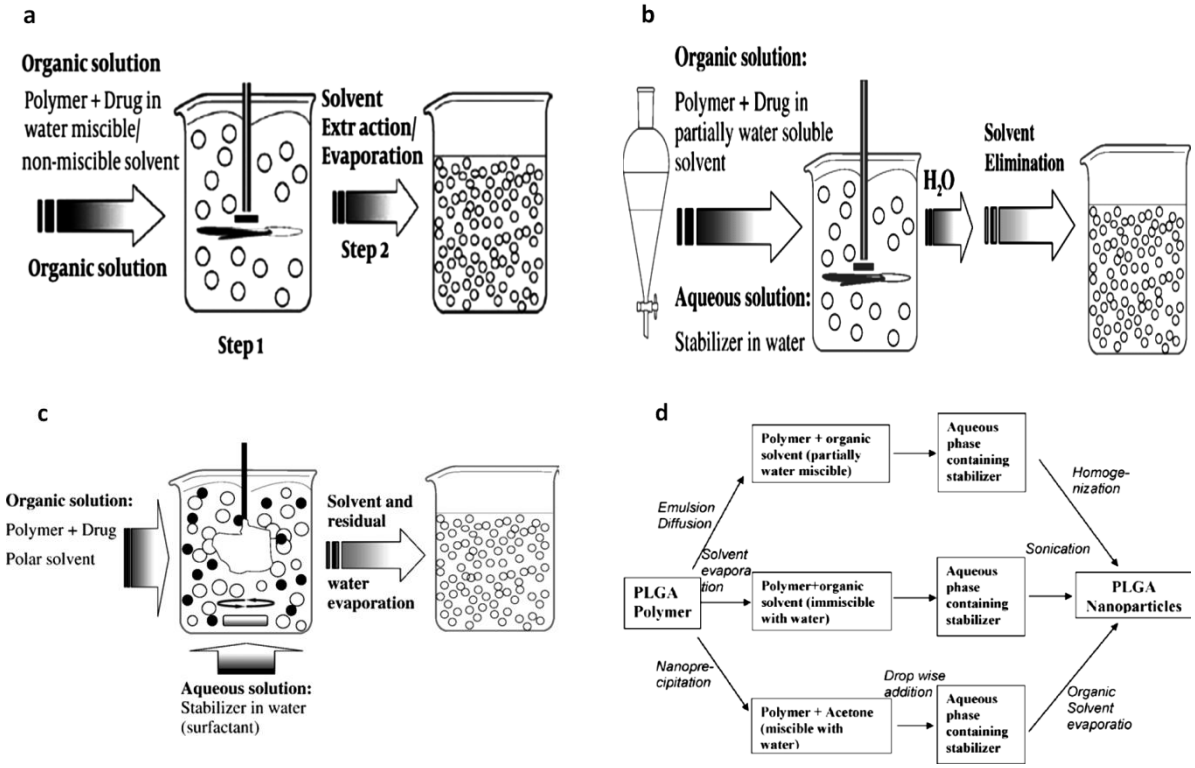

Figure 1.3: Schematic Representation of Different Preparation Methods of PLGA NPs. (a) Emulsification- Solvent Evaporation Technique. (b) Emulsification – Solvent Diffusion Technique. (c) Solvent Displacement Technique. (d) Summary of the three methods.[52,53] (Reproduced with Permission "Appendix V")

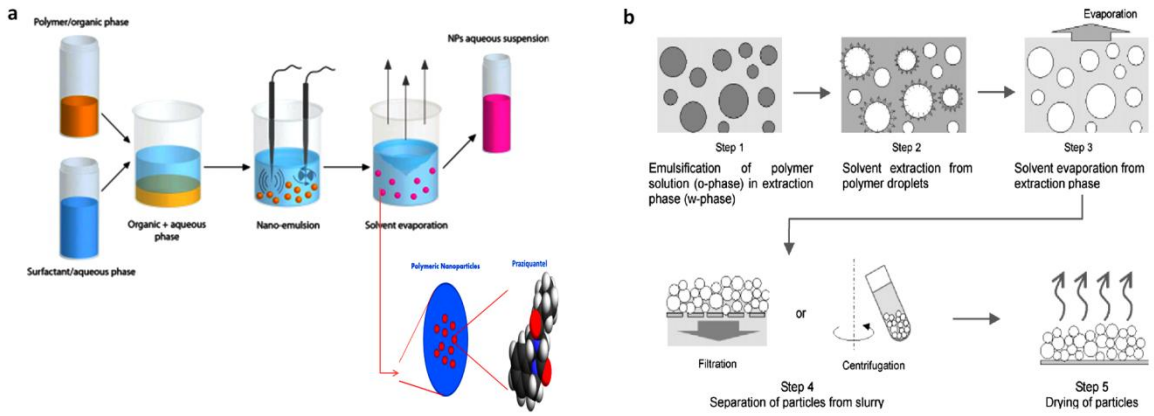

Figure 1.4: Schematic Representation of the used Single (O/W) Emulsion- Solvent Evaporation Method for Preparation of PZQ- loaded PLGA NPs . (a) Scheme of the Different Steps[62], (b) Sketch illustrating the Formation of NPs.[57] (Reproduced with Permission "Appendix V")

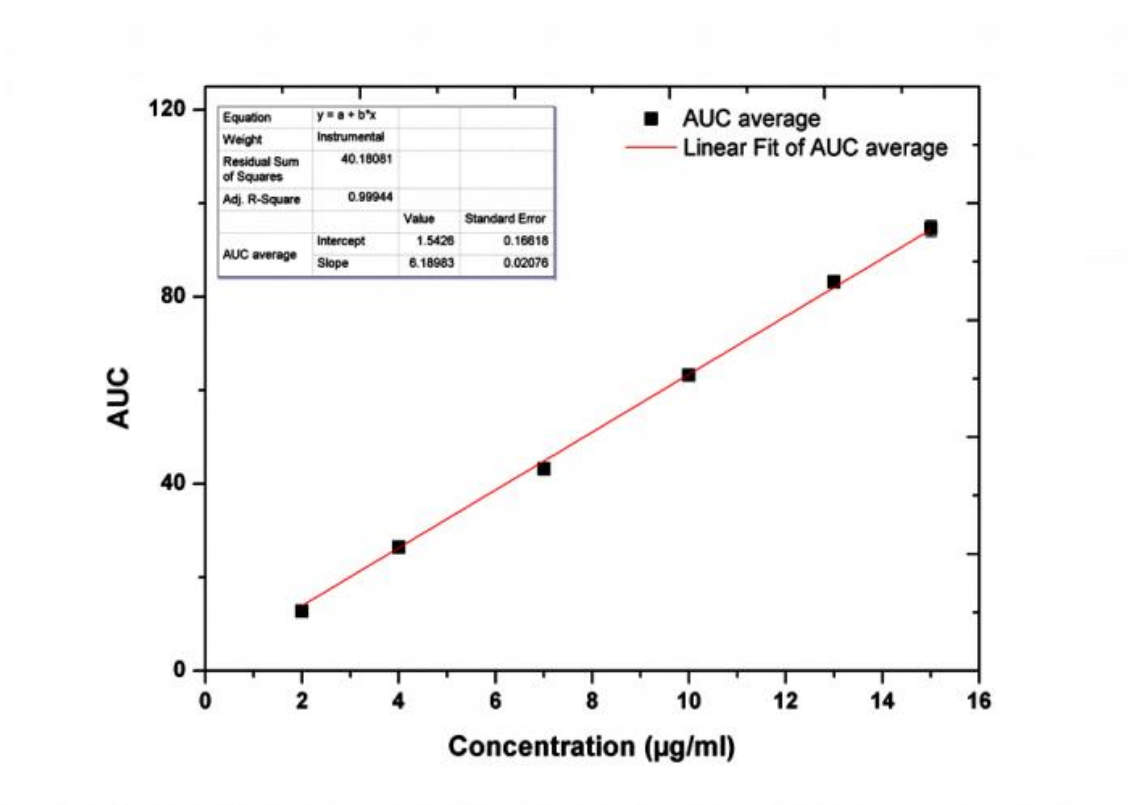

Figure 1.5: Praziquantel Standards Calibration Curve Constructed Using HPLC Method

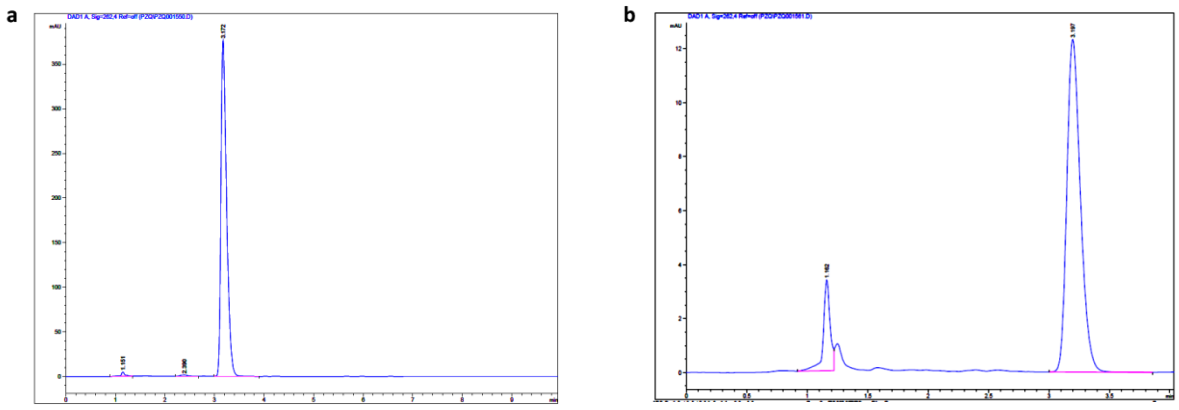

Figure 1.6: HPLC Chromatogram Showing (a) the Characteristic Peak of PZQ at Retention Time 3.172 minutes (PZQ Standard 500µg/ml), (b) PLGA Separation at Retention Time around 1.2 minutes and PZQ reading at Retention Time around 3.2 minutes Indicating No interference between the drug and the polymer at Wavelength 262nm.

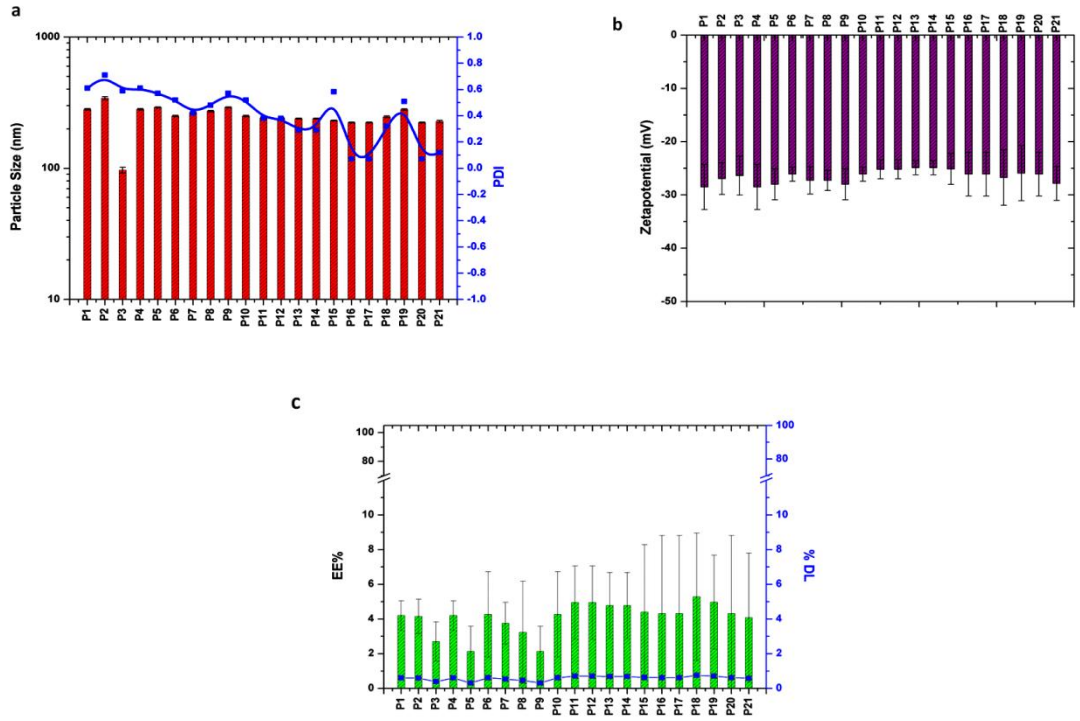

Figure 1.7: Bar Charts Comparing Between the Various Formulations Prepared in the Preliminary Study (Study I). (a) Particle Diameter, nm and Size Distribution, PDI. (b) Zetapotential, mV. (c) Encapsulation Efficiency and Drug Loading %. Data were represented as Mean±SD (n=3)

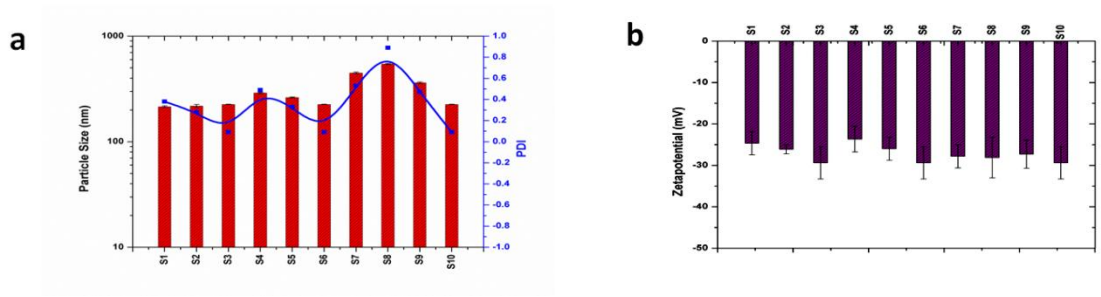

Figure 1.8: Bar Charts Comparing Between the Various Formulations Prepared in the Screening Phase (Study II). (a) Particle Diameter, nm and Size Distribution, PDI. (b) Zetapotential, mV. Data were represented as Mean±SD (n=3)

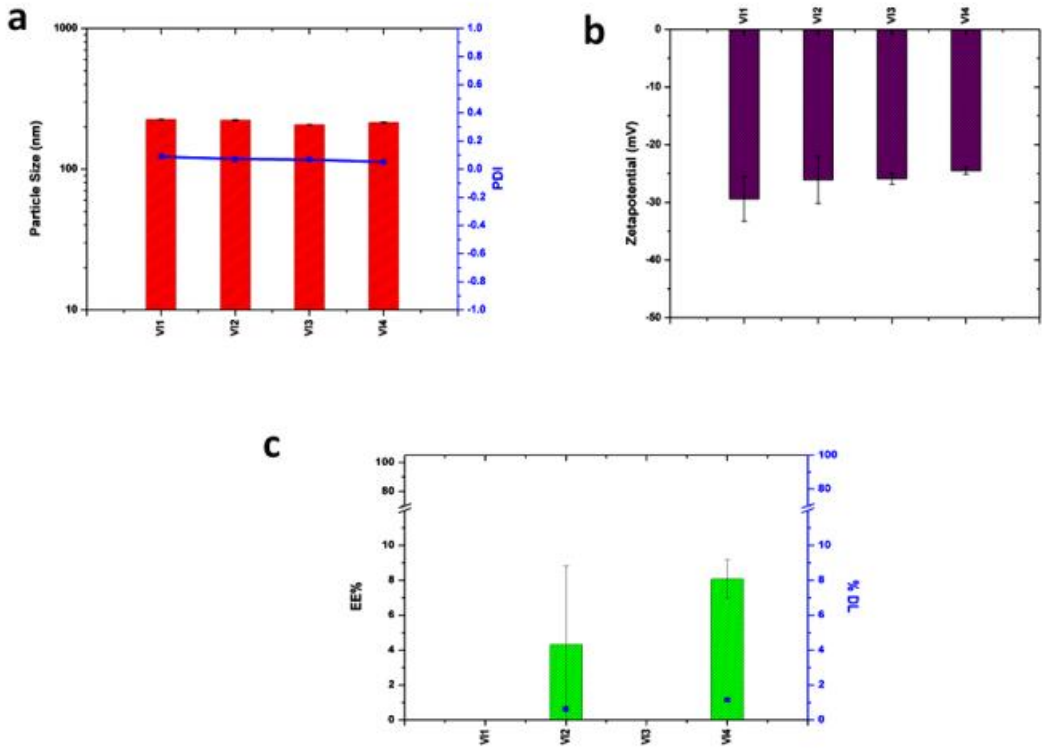

Figure 1.9: Bar Charts Comparing Between the Various Formulations Prepared in the Validation Phase I (Study III). (a) Particle Diameter, nm and Size Distribution, PDI. (b) Zetapotential, mV. (c) Encapsulation Efficiency and Drug Loading %. Data were represented as Mean±SD (n=3)

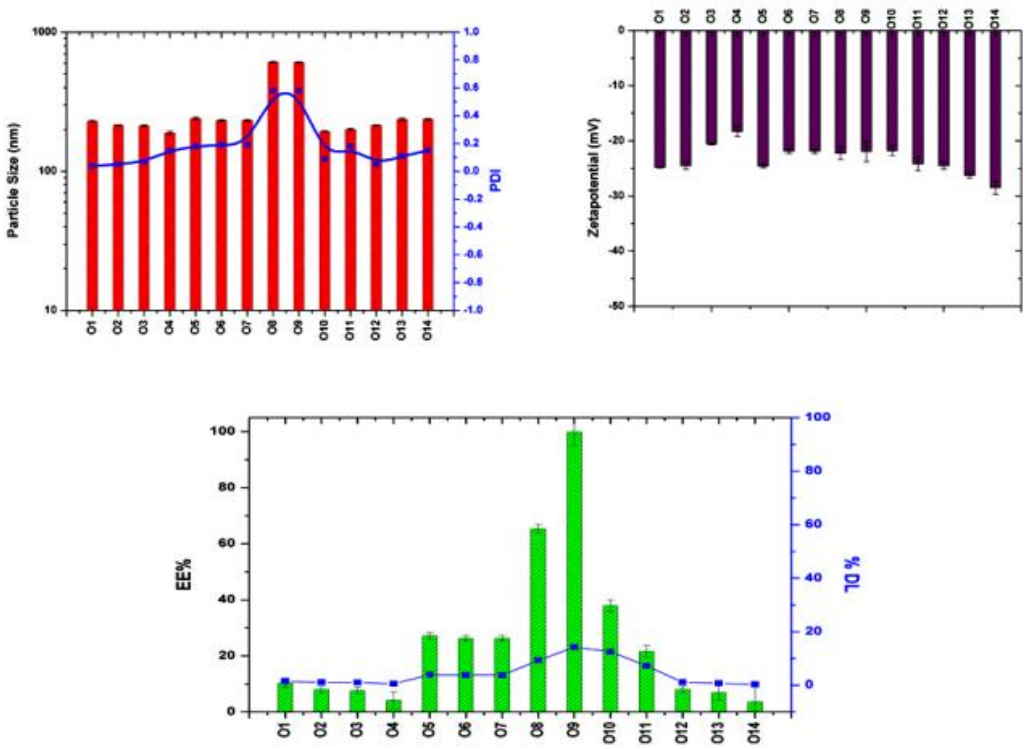

Figure 1.10: Bar Charts Comparing Between the Various Formulations Prepared in the Optimization Phase (Study IV). (a) Particle Diameter, nm and Size Distribution, PDI. (b) Zetapotential, mV. (c) Encapsulation Efficiency and Drug Loading %. Data were represented as Mean±SD (n=3)

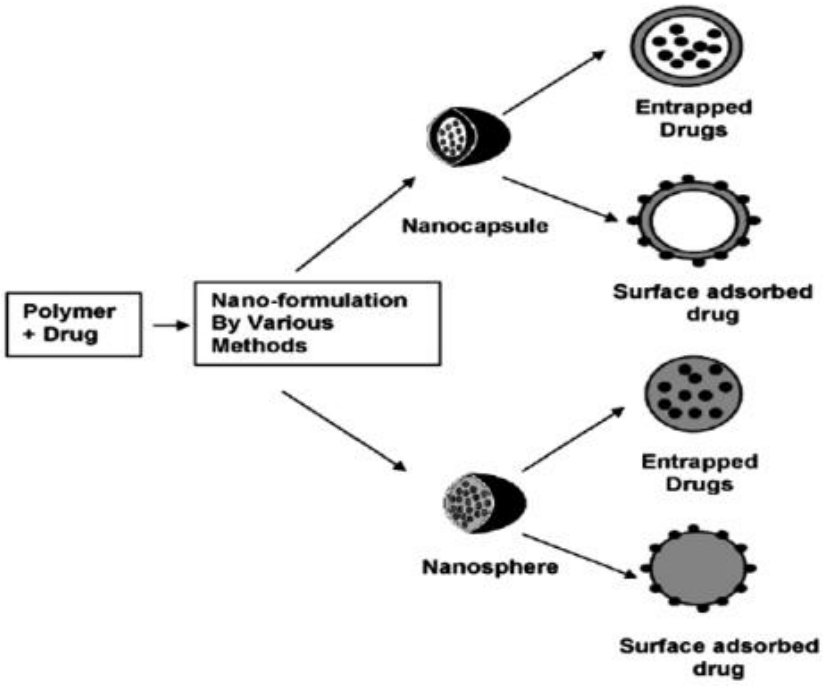

Figure 1.11: Type of biodegradable nanoparticles: According to the structural organization biodegradable nanoparticles are classified as nanocapsule, and nanosphere. Most of the drug is entrapped inside the polymer and small part of it is adsorbed on the surface.[52] (Reproduced with Permission "Appendix V")

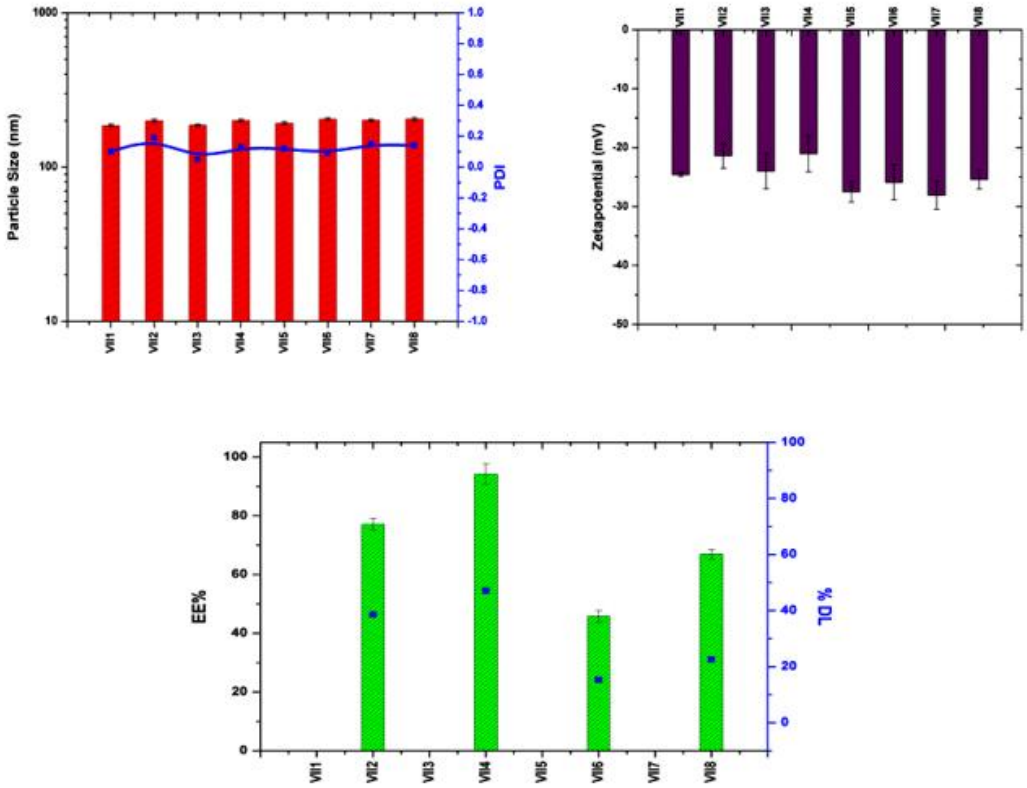

Figure 1.12: Bar Charts Comparing Between the Various Formulations Prepared in the Validation Phase II (Study V). (a) Particle Diameter, nm and Size Distribution, PDI. (b) Zetapotential, mV. (c) Encapsulation Efficiency and Drug Loading %. Data were represented as Mean±SD (n=3)

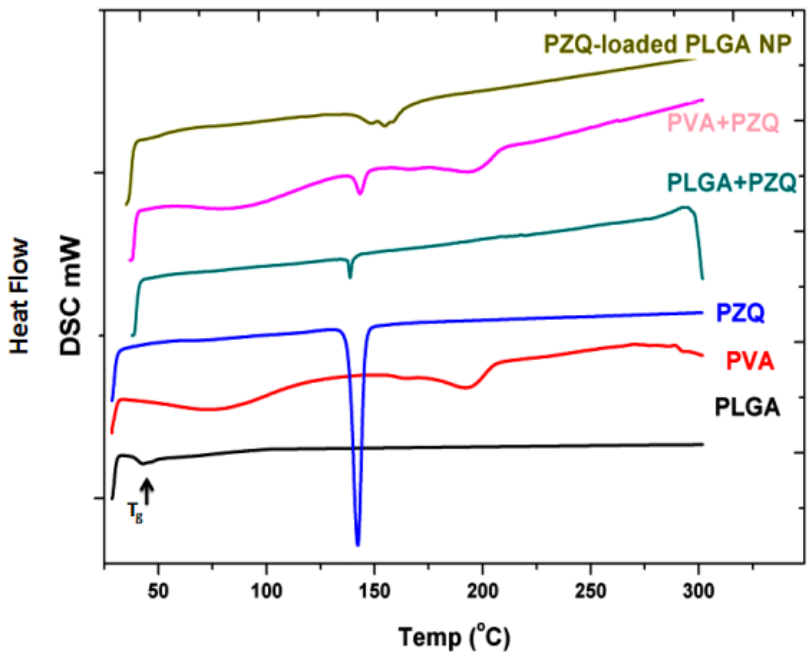

Figure 1.13: DSC Thermogram of Pure PLGA, PVA, Free PZQ, Physical Mixture of PZQ and PLGA, Physical Mixture of PZQ and PVA and PZQ- loaded PLGA NPs.

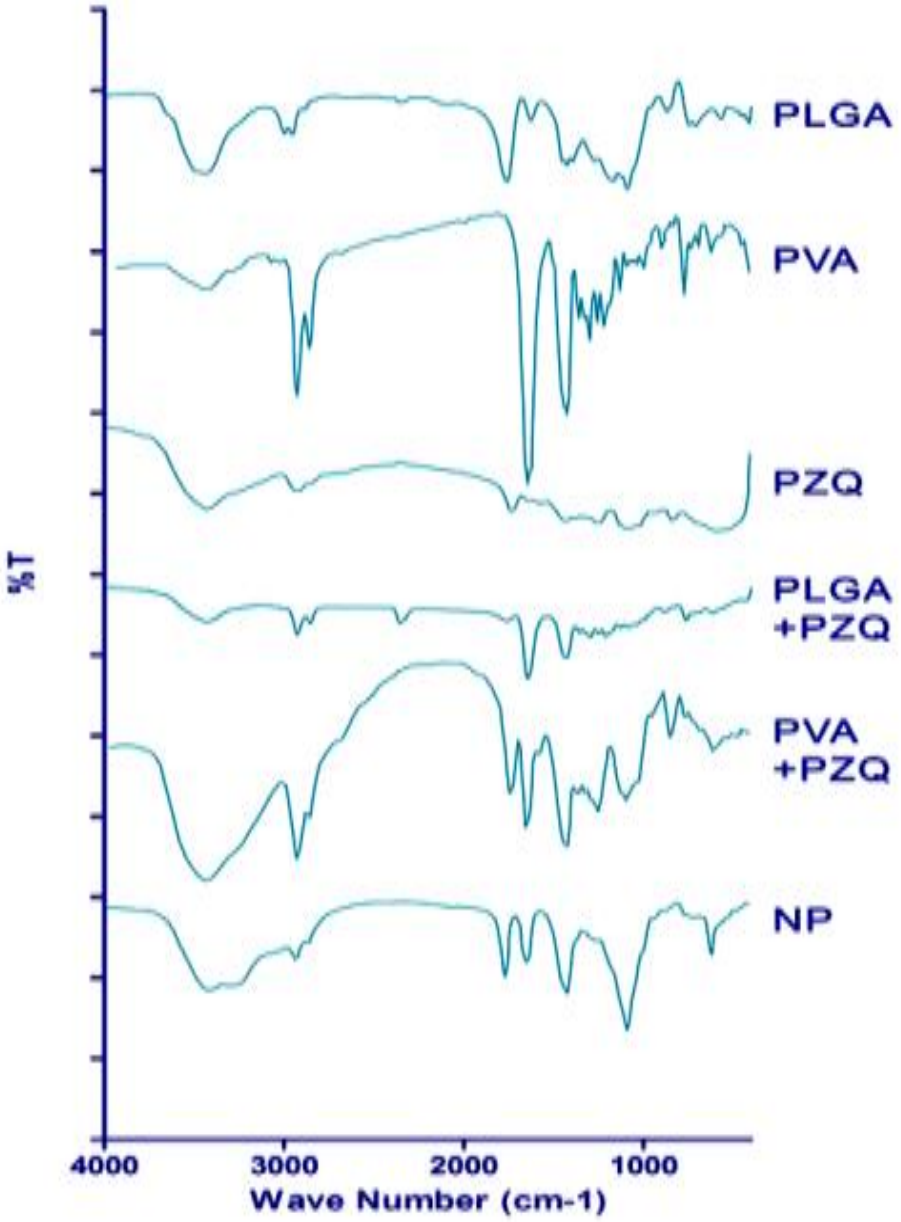

Figure 1.14: FTIR Spectrum of Pure PLGA, PVA, Free PZQ, Physical Mixture of PZQ and PLGA, Physical Mixture of PZQ and PVA and PZQ- loaded PLGA NPs.

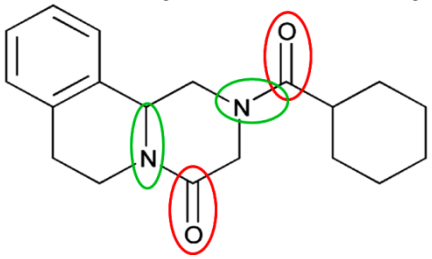

Figure 1.15: Chemical Structure of Praziquantel. [133]

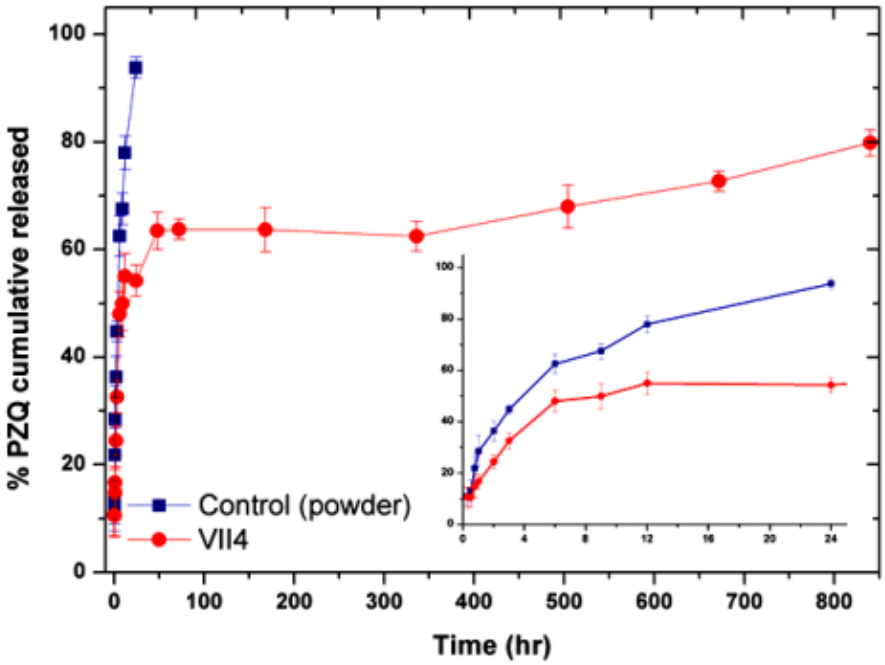

Figure 1.16: Release Profile of PZQ from the optimized Formula VII4 in Comparison with Pure PZQ Powder. Data was present as Mean±SD of triplicate experiment.

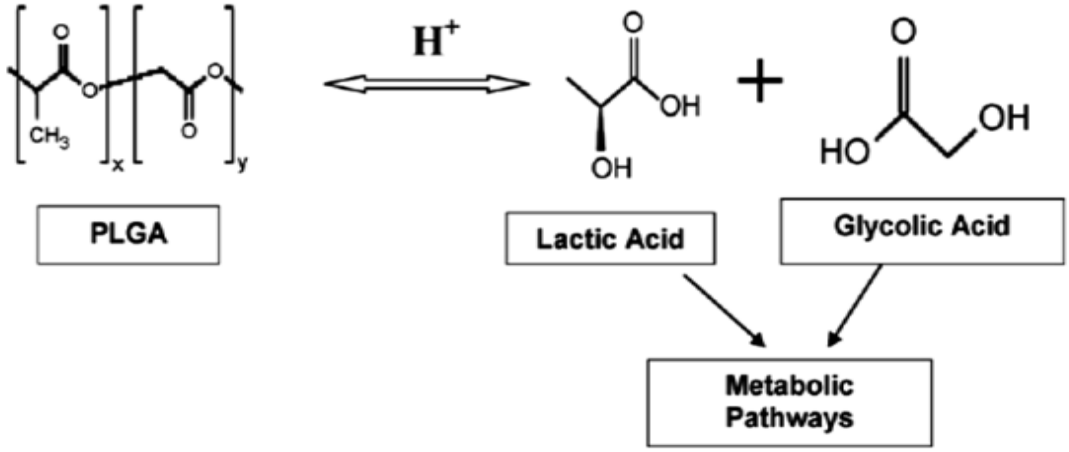

Figure 1.17: Degradation and Hydrolysis of PLGA Nanoparticles. Biologically, the degradation products, lactic and glycolic acids, will be metabolized in Citric Acid "Krebs" Cycle.[52] (Reproduced with Permission "Appendix V")

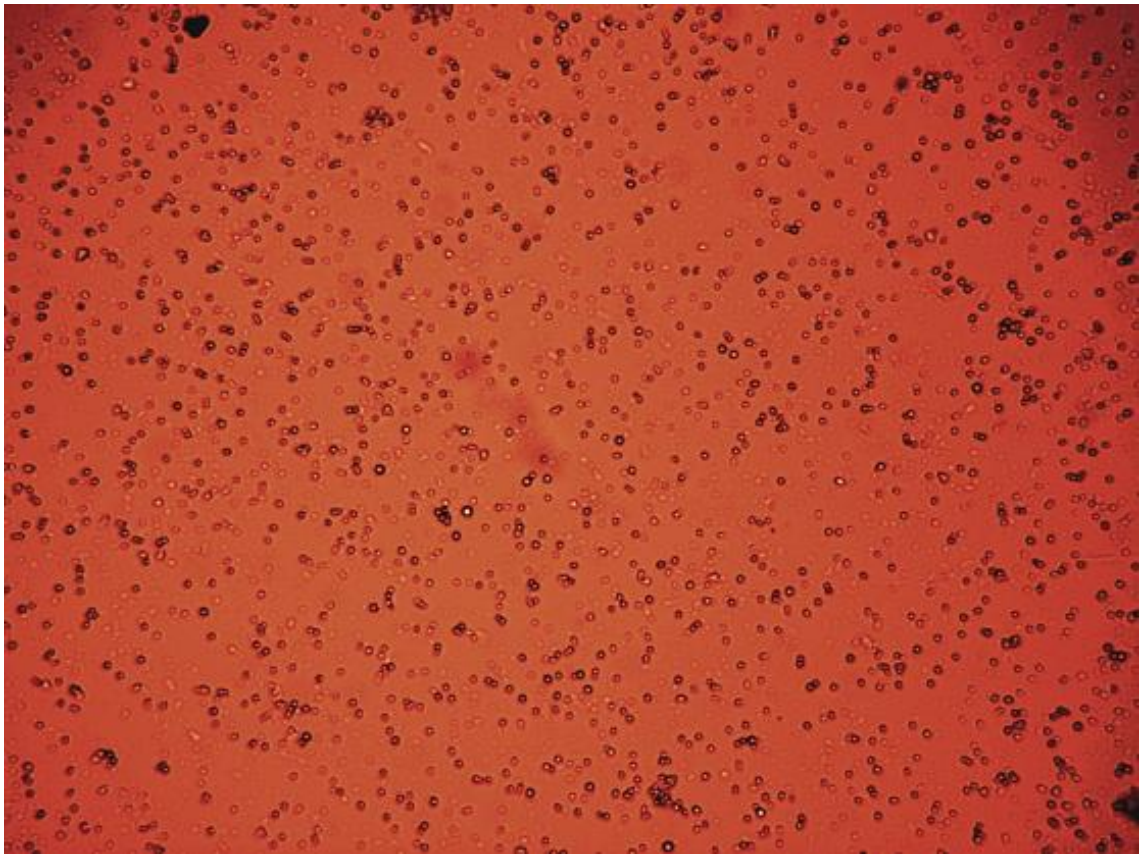

Figure 1.18: Optical Microscope. (Optimum Formula **VII4**, 40X Magnification)

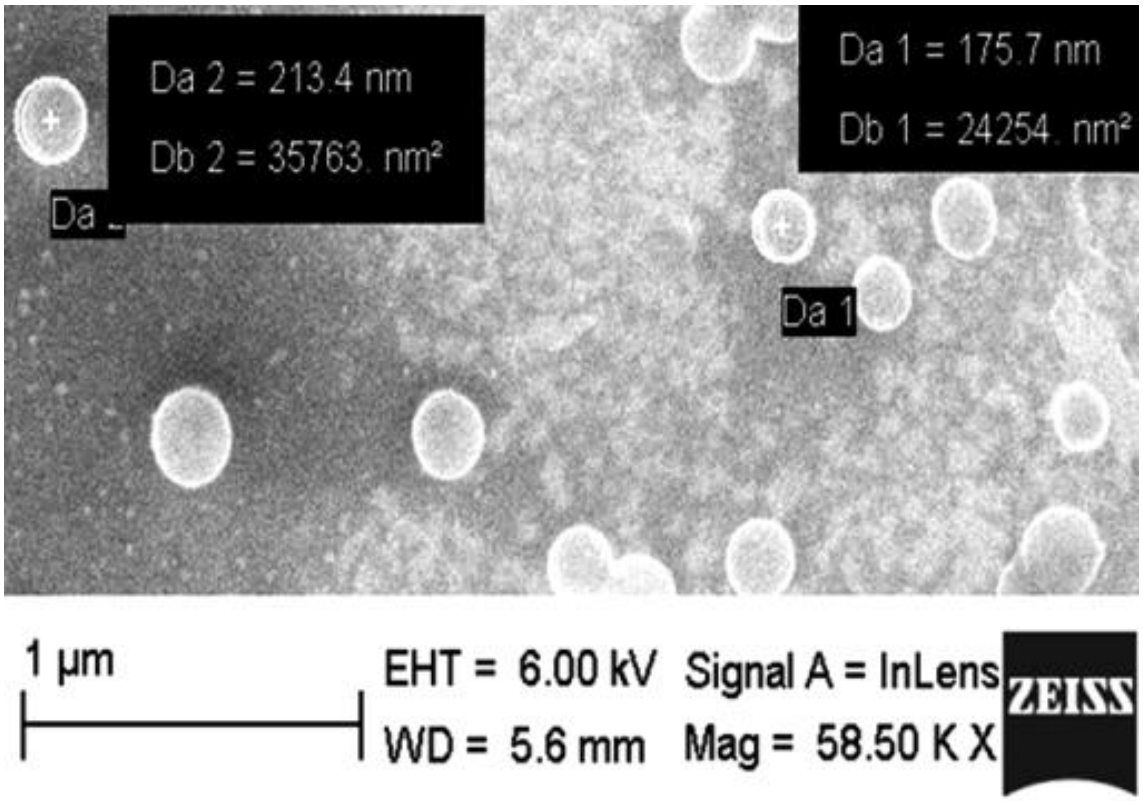

Figure 1.19: Scanning Electron Micrograph of the optimum PZQ- loaded PLGA NPs Formula (**VII4**).

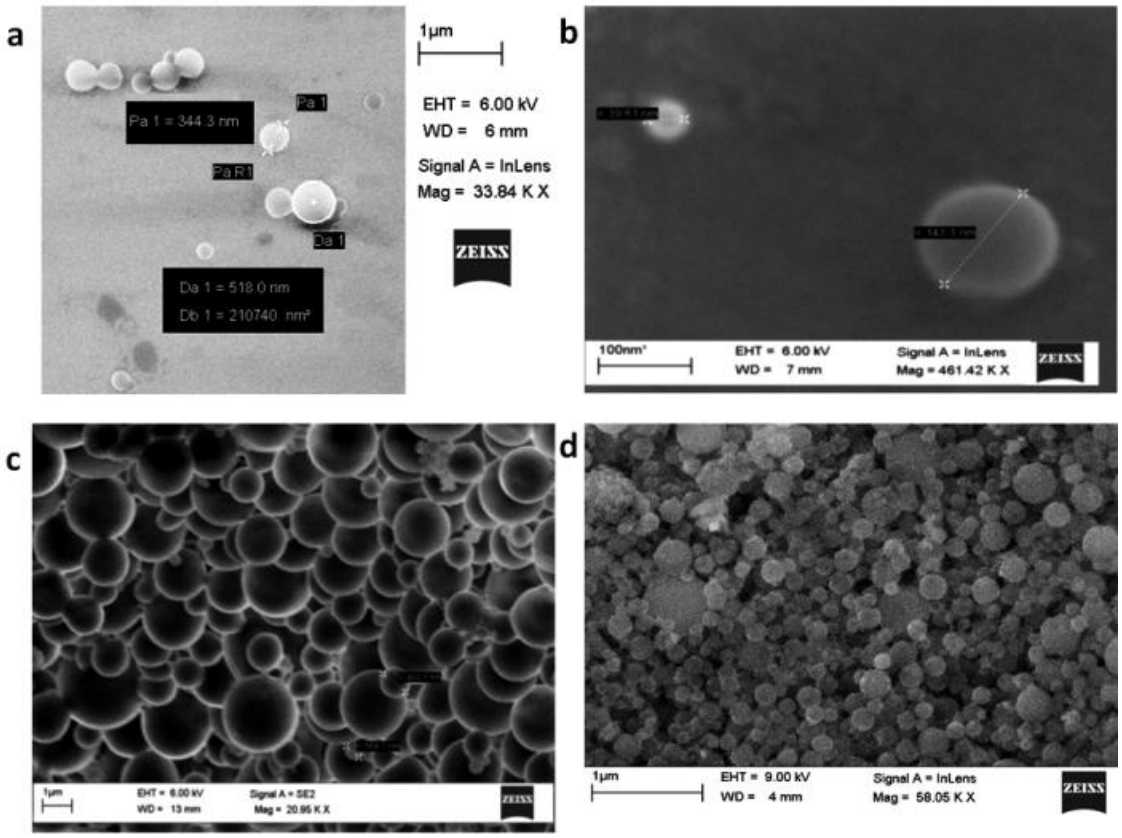

Figure 1.20: Scanning Electron Micrograph of (**a**) Formula **S7** prepared using Poloxamer 407, (**b**) Formula **P3** Prepared by Nanoprecipitation Using Acetone as Organic Solvent, (**c**) Formula **O9** prepared Using 20% Ammonium Sulphate in External Phase, and (**d**) Lyophilized NPs of Formula **P21**.

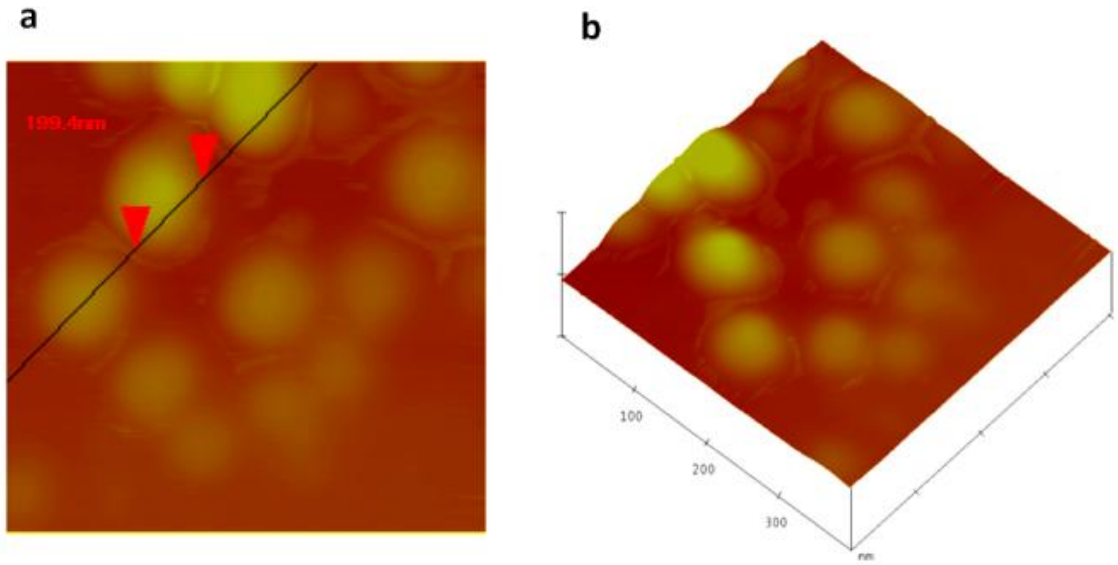

Figure 1.21: Two-dimensional (**a**) and Three-dimensional (**b**) AFM topographic images of Optimum PZQ- loaded PLGA Formula **VII4**.

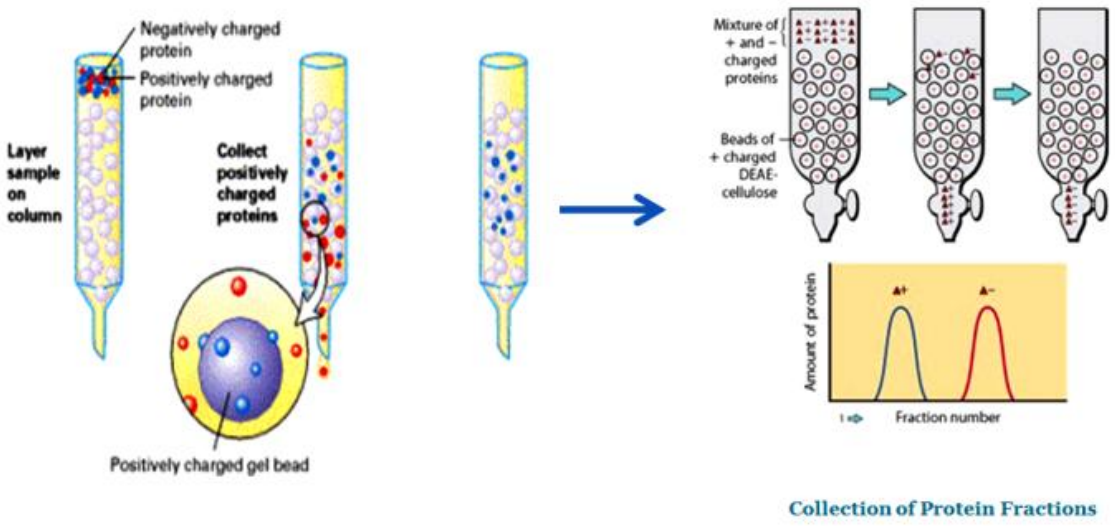

Figure 2.1: Basic Principle of Ion exchange chromatography. (Modified from[134])

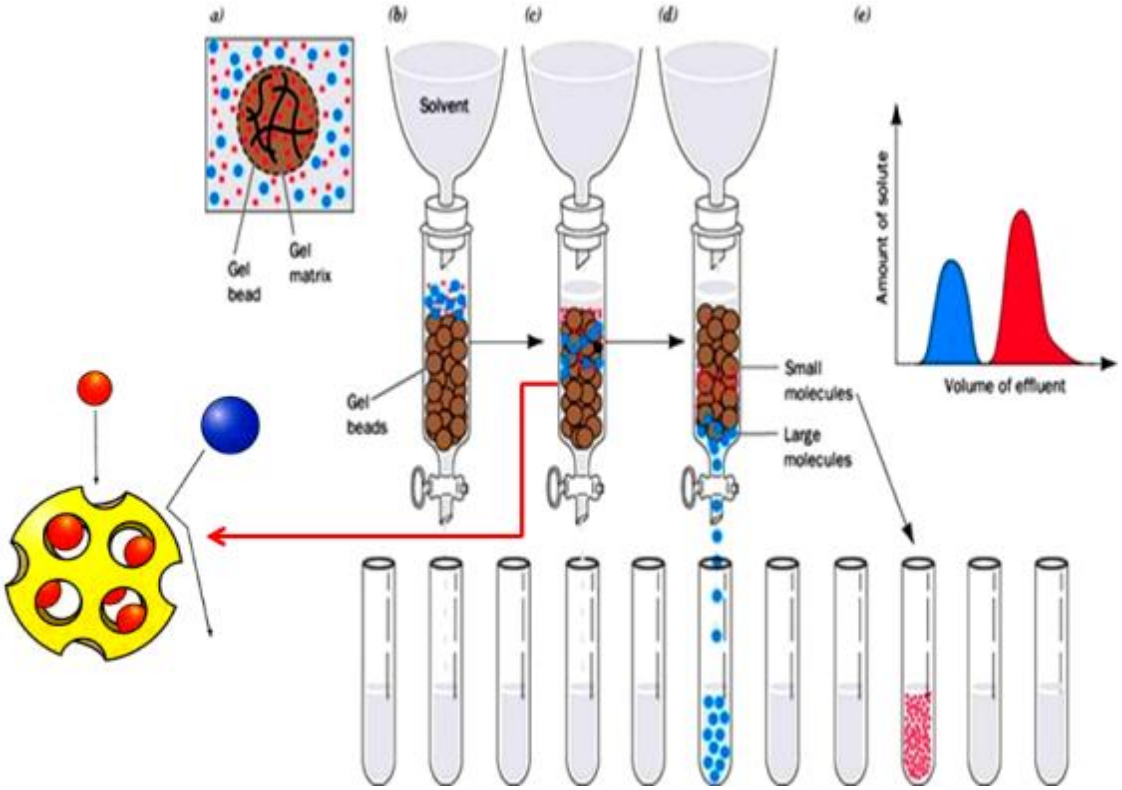

Figure 2.2: Basic Principle of Gel Filtration (Size Exclusion) Chromatography. (Modified from [135])

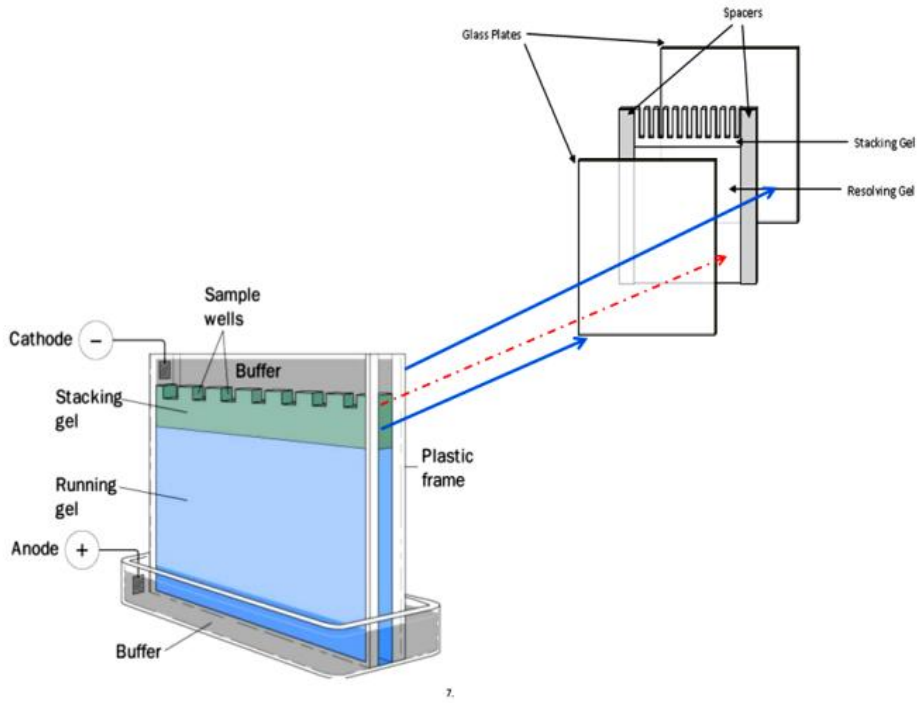

Figure 2.3: Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS –PAGE) Gel Setup. (Modified from[136])

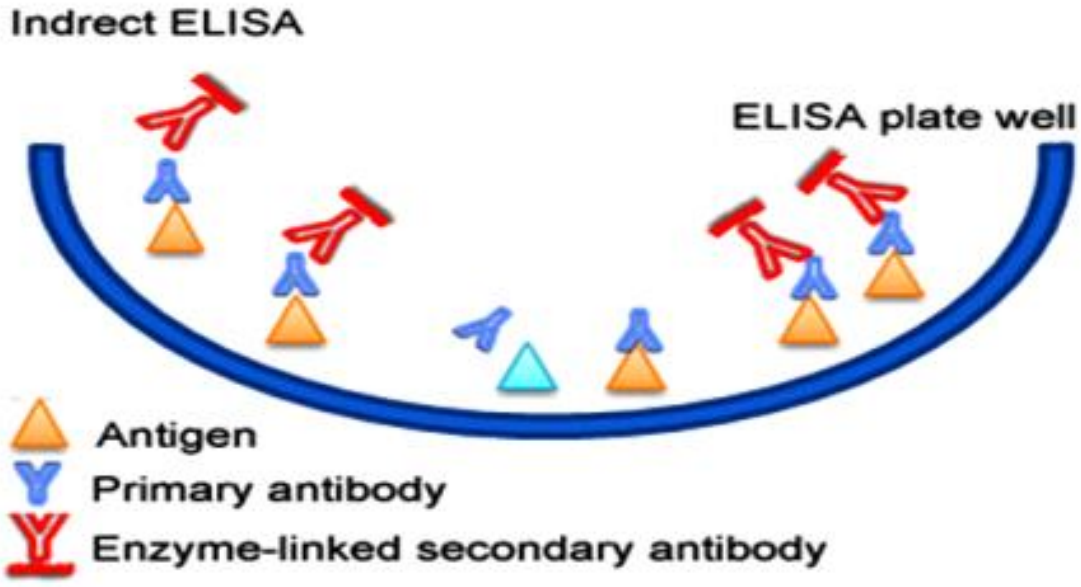

Figure 2.4: Basic Principle of Indirect ELISA. [137]

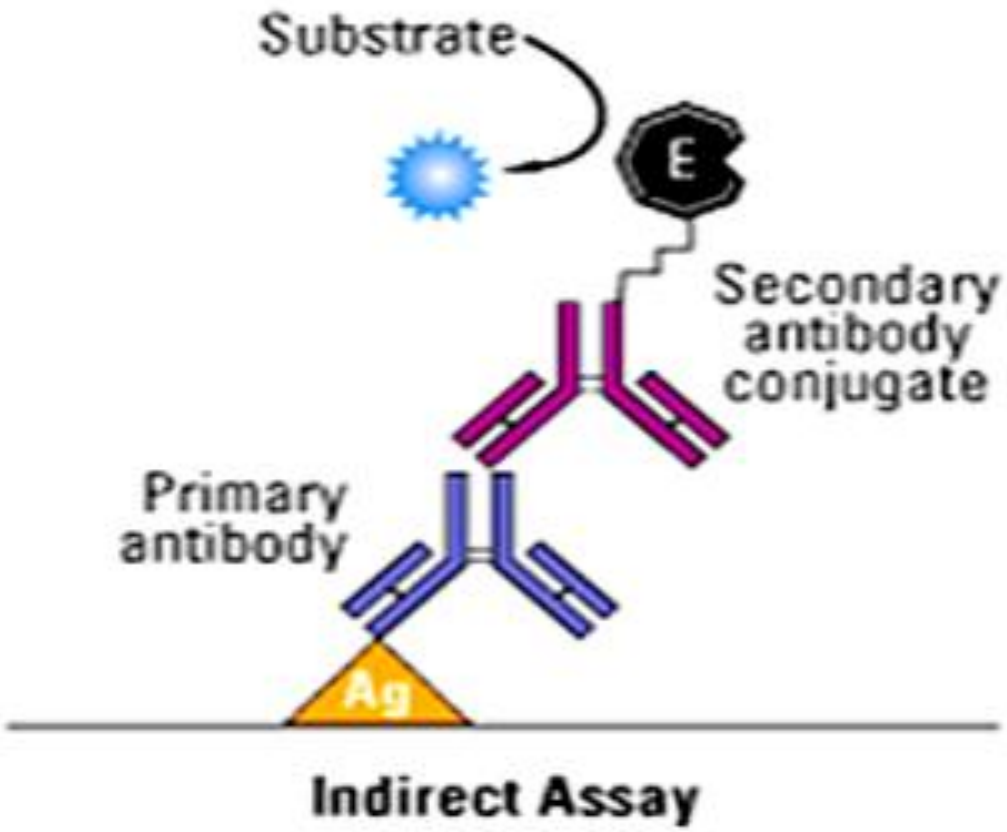

Figure 2.5: Basic Principle of Dot Blot (Indirect- Type Immunoassay). [138]

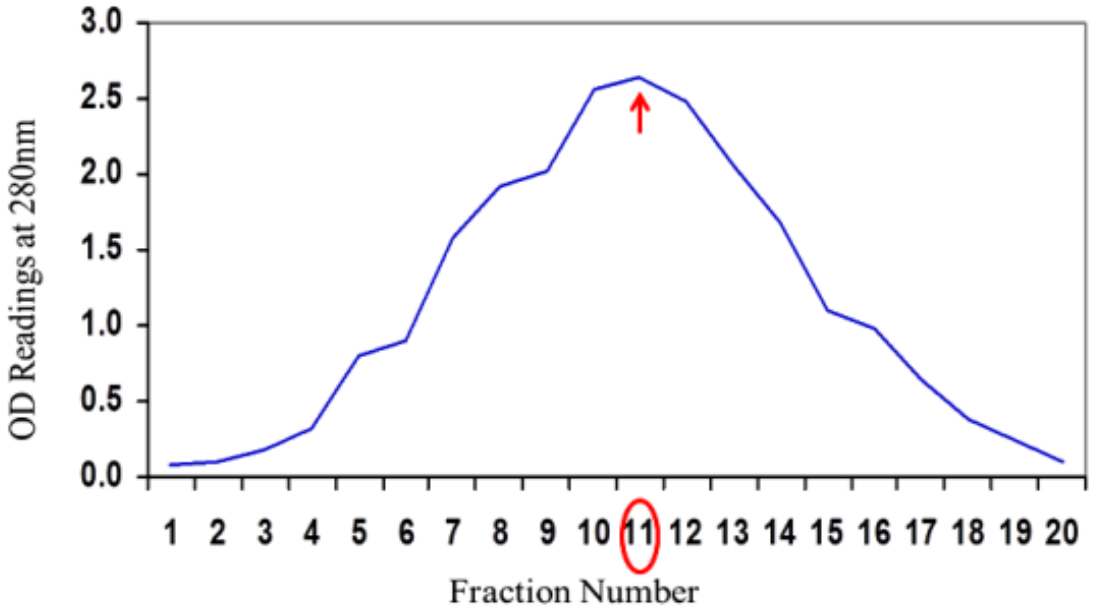

Figure 2.6: Fractions of Antigens after Purification of E/S Crude Products by Ion Exchange Chromatography on DEAE Sephadex A-50 Column.

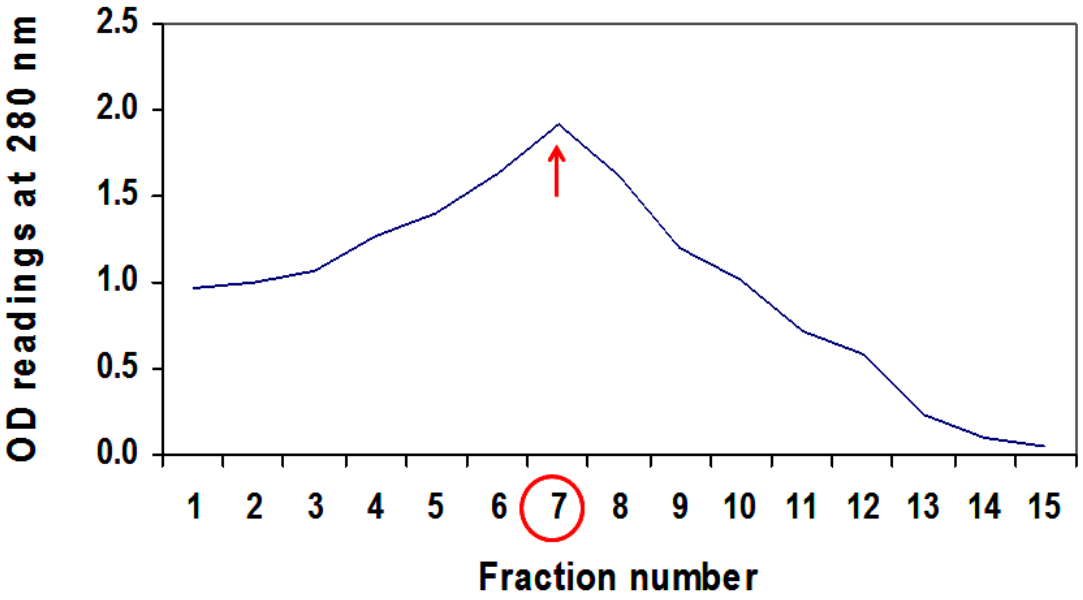

Figure 2.7: Fractions of Antigens after Gel Filtration Chromatography on Sephacryl S-100 HR Column.

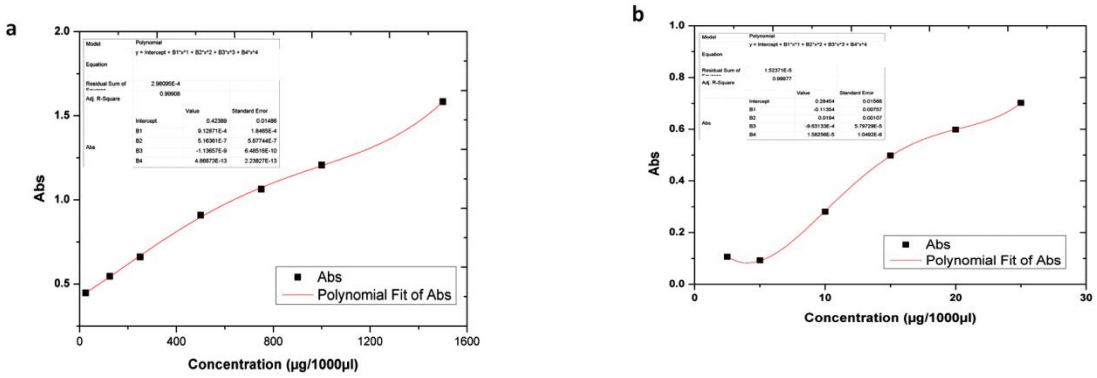

Figure 2.8: BSA Calibration Curve. (**a**) Concentration Range: 0-2000µg/ml, (**b**) Concentration Range: 0-25g/ml.

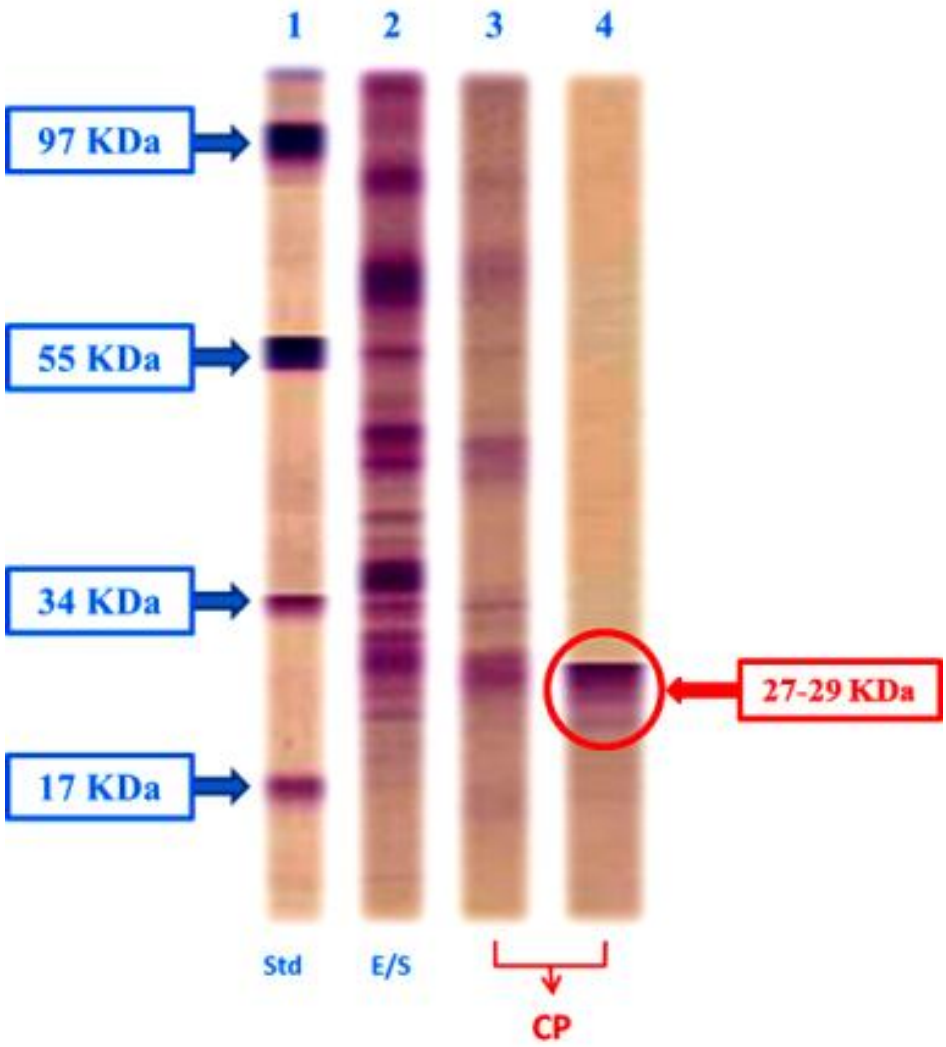

Figure 2.9: SDS-PAGE Gel Profile of Adult *S. haematobium* Worm Antigen Fractions Eluted from Different Chromatography Columns

1: Low molecular weight standard protein.
2: Crude E/S product.
3: Fraction containing target CP antigen partially purified on DEAE Sephadex A-50 column.
4: Fraction containing target purified CP antigen eluted from Sephacryl S-100 HR column.

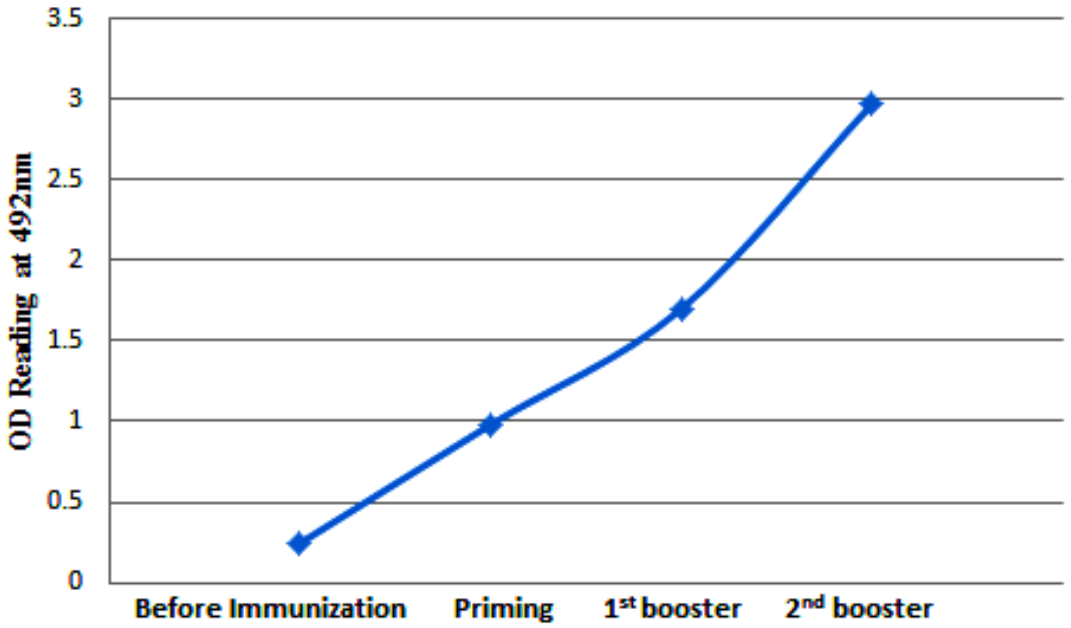

Figure 2.10: Reactivity of immunized rabbit anti- *S. haematobium* CP antisera (pAb) against *S. haematobium* CP Antigen by Indirect ELISA.

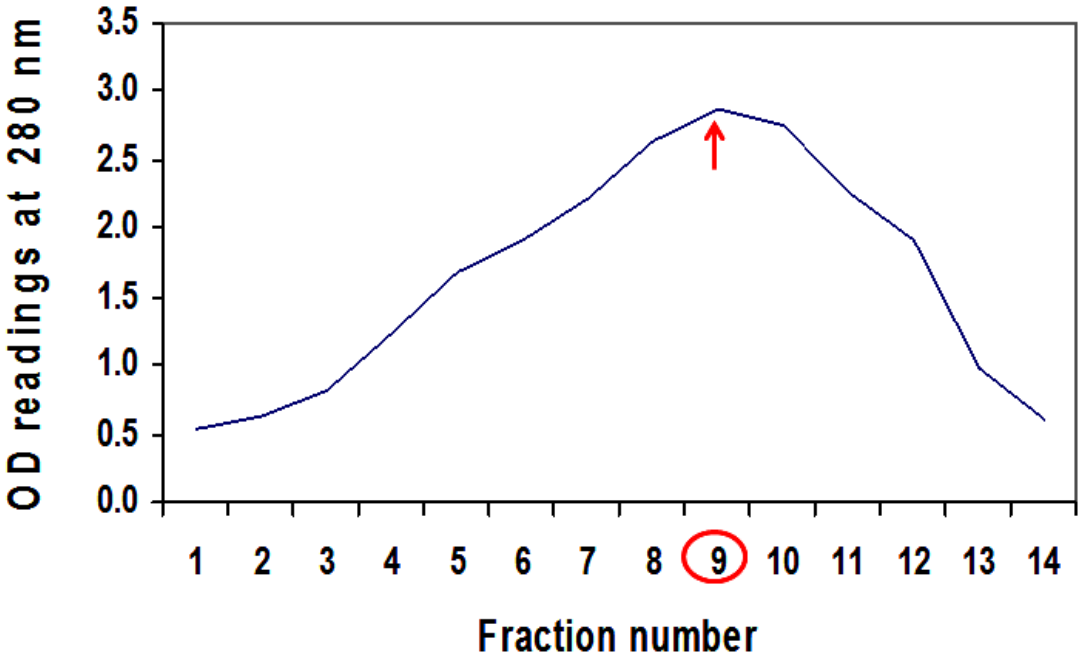

Figure 2.11 : Fractions of Rabbit Anti- CP pAb after Purification by Ion Exchange Chromatography on DEAE Sephadex A-50 Column.

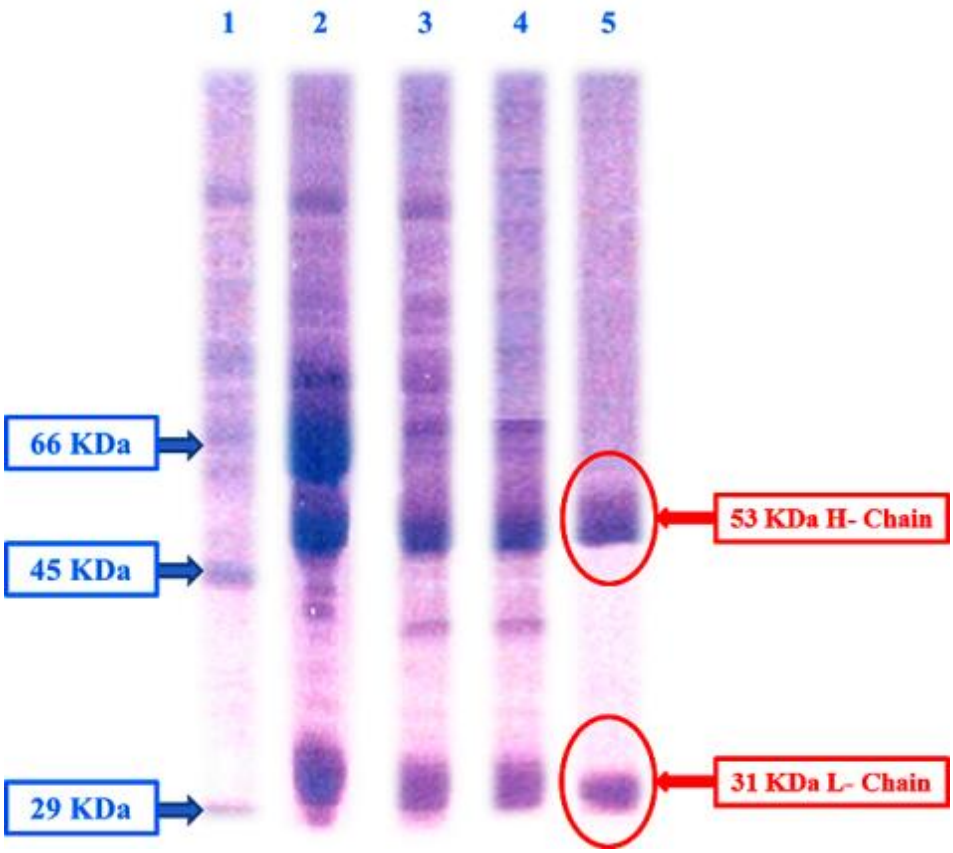

Figure 2.12: SDS-PAGE Gel Profile of Anti- *S. haematobium* IgG pAb before and after purification

1: Low molecular weight of standard protein.
2: Crude anti- *S. haematobium* CP pAb (before purification).
3: Precipitated proteins after 50% ammonium sulfate treatment.
4: Partially purified IgG-pAb after 7% caprylic acid treatment.
5: Purified IgG-pAb after ion exchange chromatography.

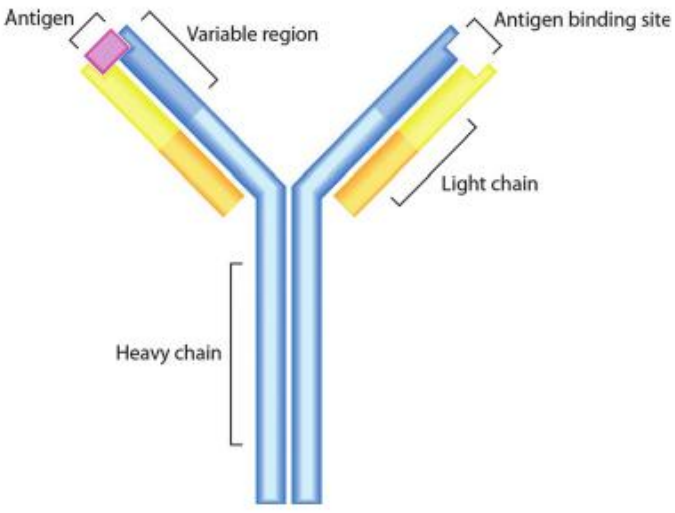

Figure 2.13: General Structure of an Antibody.[62] (Reproduced with Permission "Appendix V")

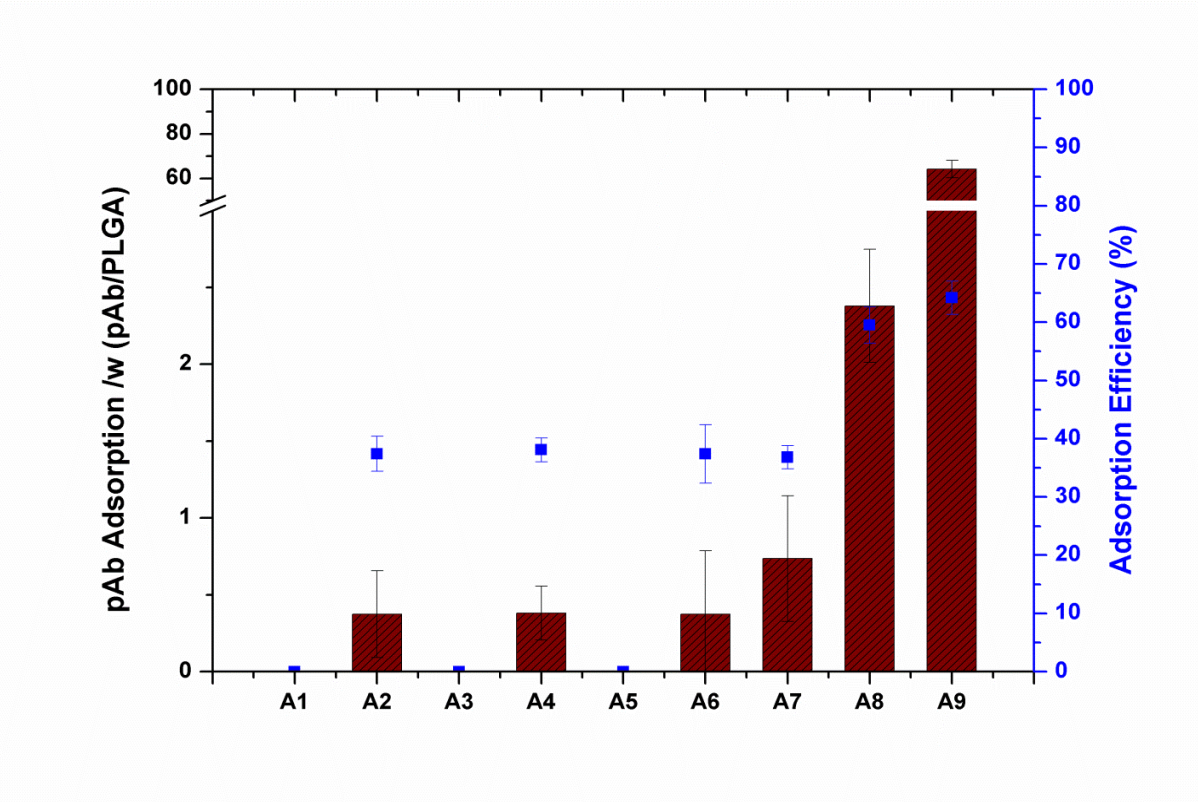

Figure 2.14: Comparison between Different Immuno-NPs Formulations Surface- Coated via pAb Adsorption Method with regards to Adsorption Efficiency % and the Amount of Adsorbed pAb per mg of PLGA.

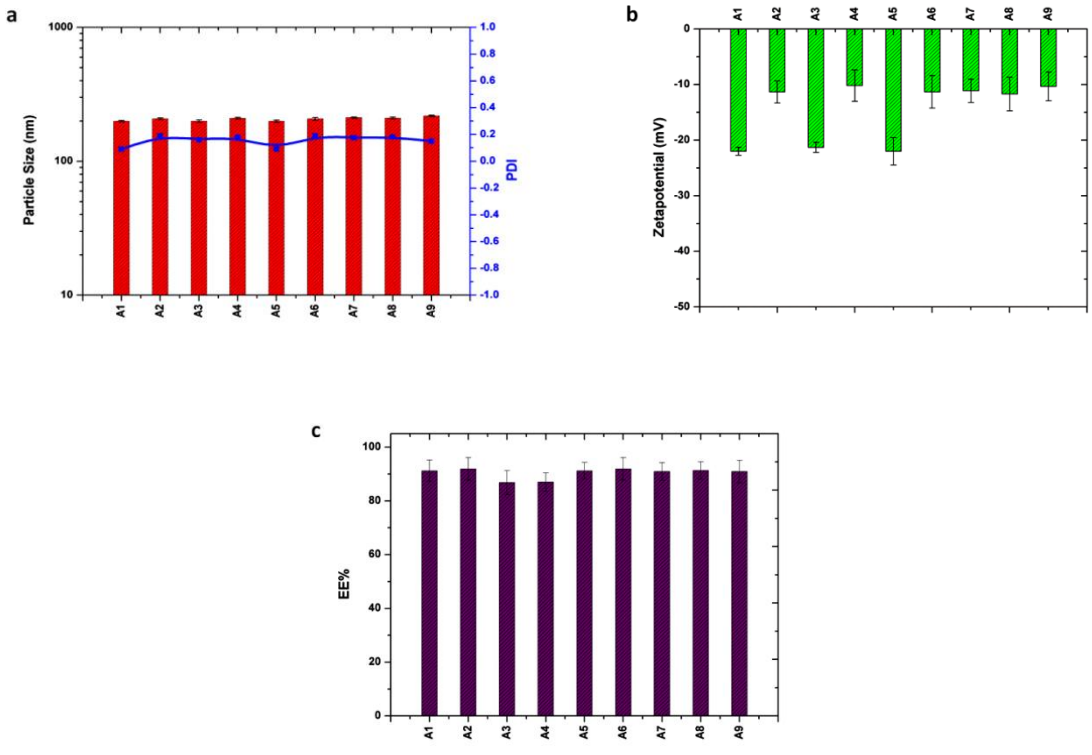

Figure 2.15: Bar Charts Comparing the Physical Properties of Various pAb-Adsorbed PLGA NPs Formulations. (**a**) Mean NPs Size "nm" and Size Distribution "PDI", (**b**) Zetapotential "mV", and (**c**) Encapsulation Efficiency %.

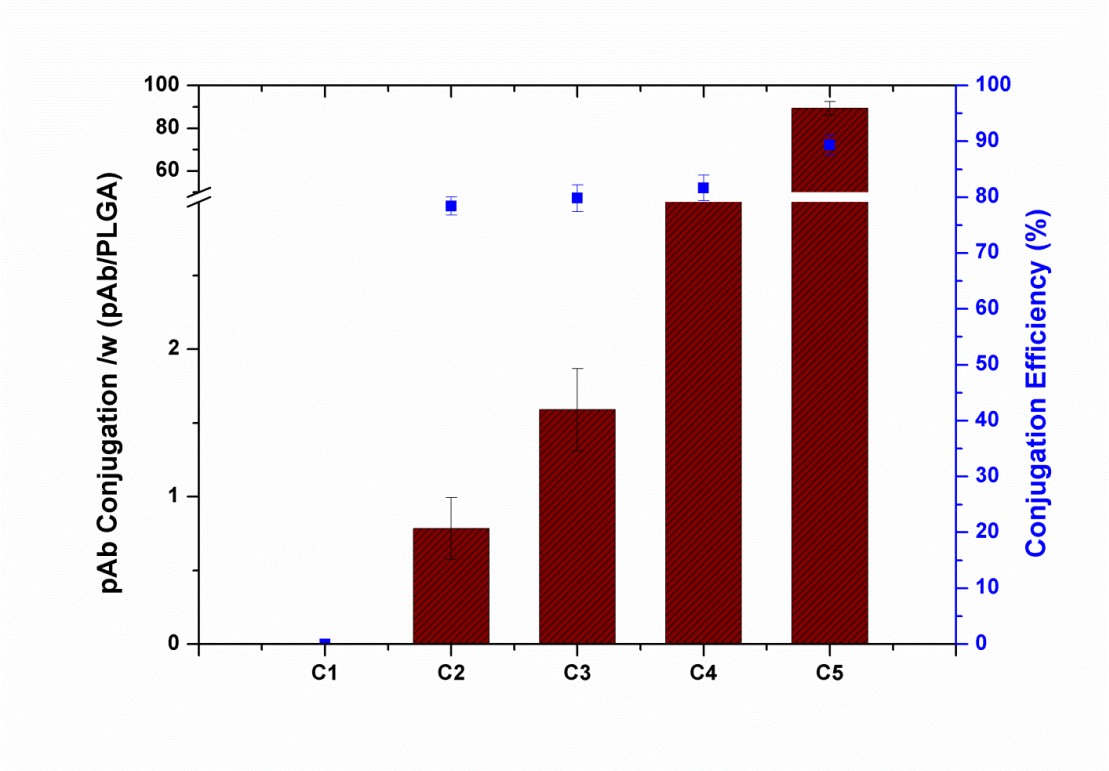

Figure 2.16: Comparison between Different Immuno-NPs Formulations Surface- Coated via pAb Chemical Conjugation Method with regards to Conjugation Efficiency % and the Amount of Conjugated pAb per mg of PLGA.

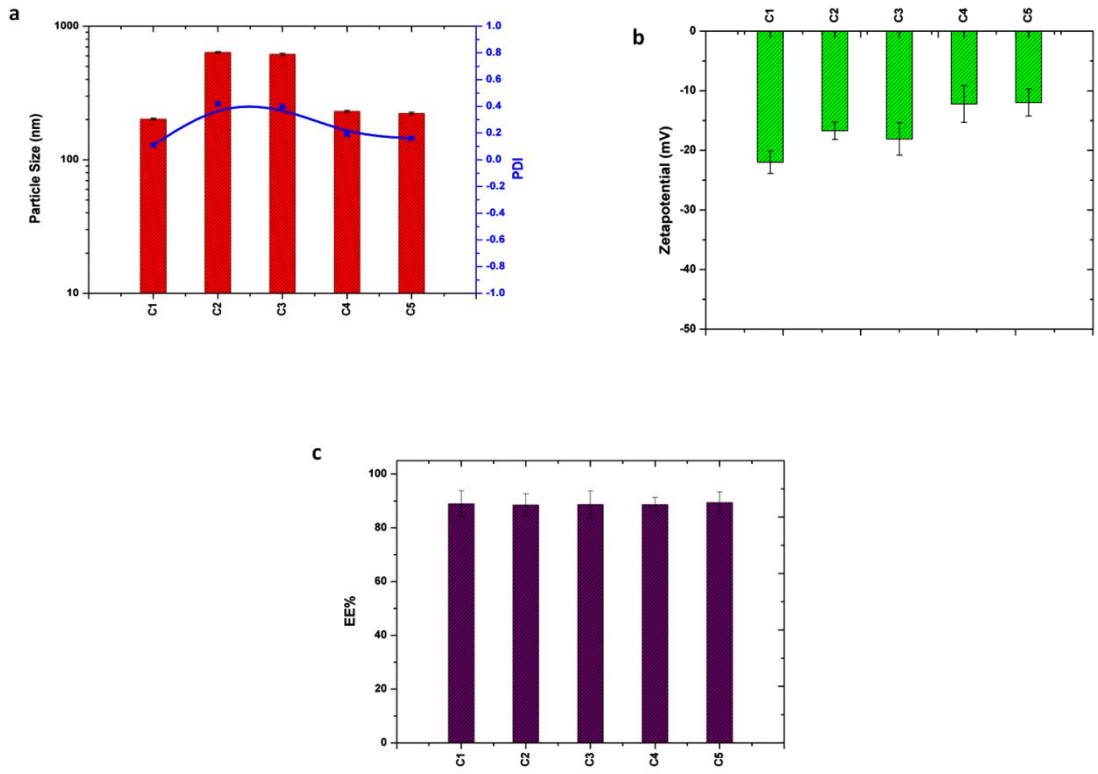

Figure 2.17: Bar Charts Comparing the Physical Properties of Various pAb-Conjugated PLGA NPs Formulations. (**a**) Mean NPs Size "nm" and Size Distribution "PDI", (**b**) Zetapotential "mV", and (**c**) Encapsulation Efficiency %.

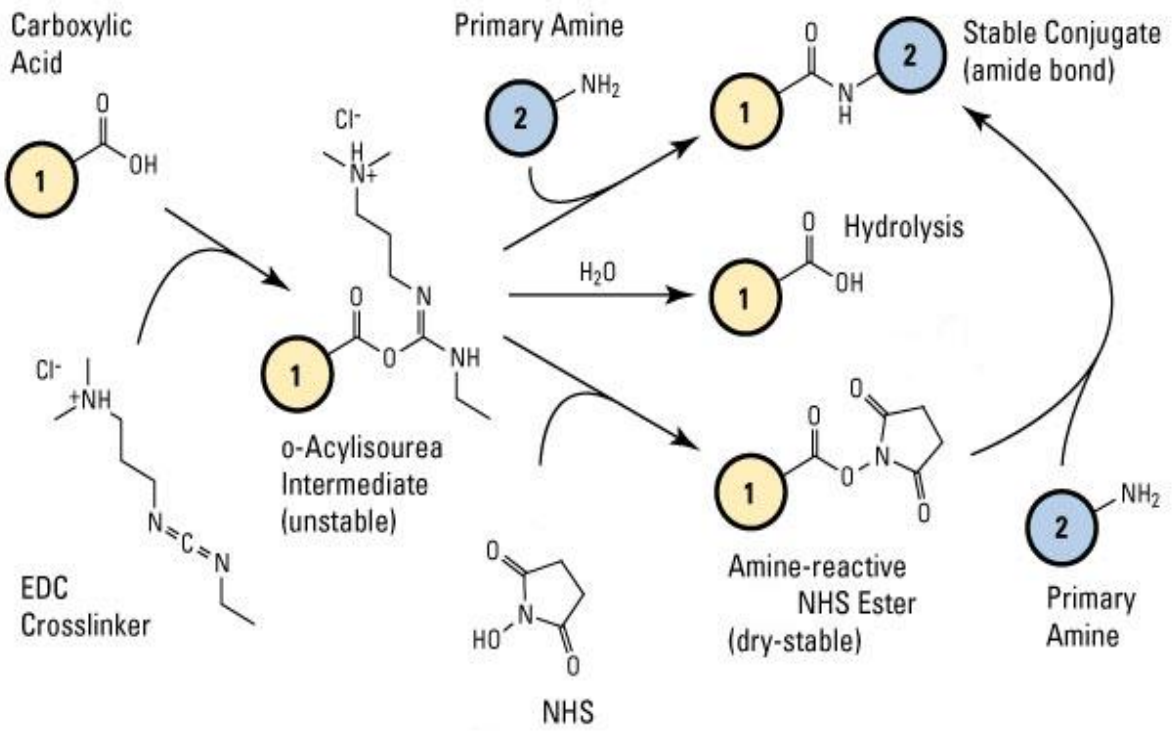

Figure 2.18: Scheme illustrating EDC/NHS Carbodiimide Crosslinking Reaction. (Modified from[139])

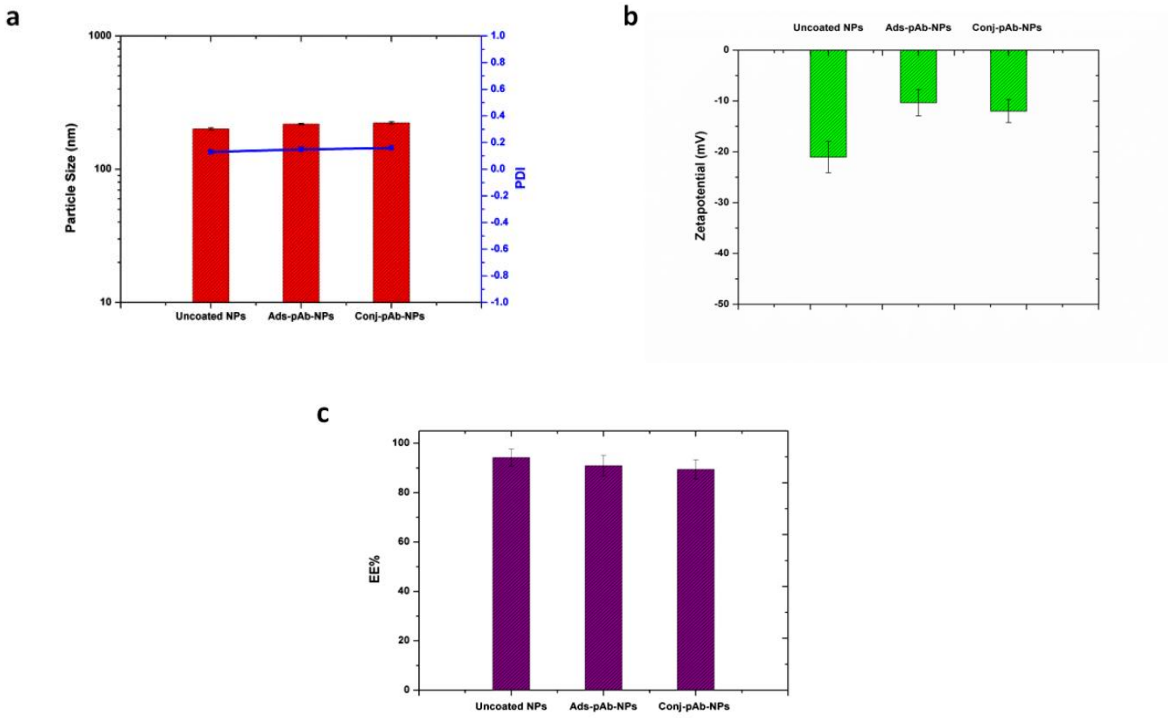

Figure 2.19: Bar Charts Comparing the Physical Properties of the Three Optimized PLGA NPs Formulations. (**a**) Mean NPs Size "nm" and Size Distribution "PDI", (**b**) Zetapotential "mV", and (**c**) Encapsulation Efficiency %.

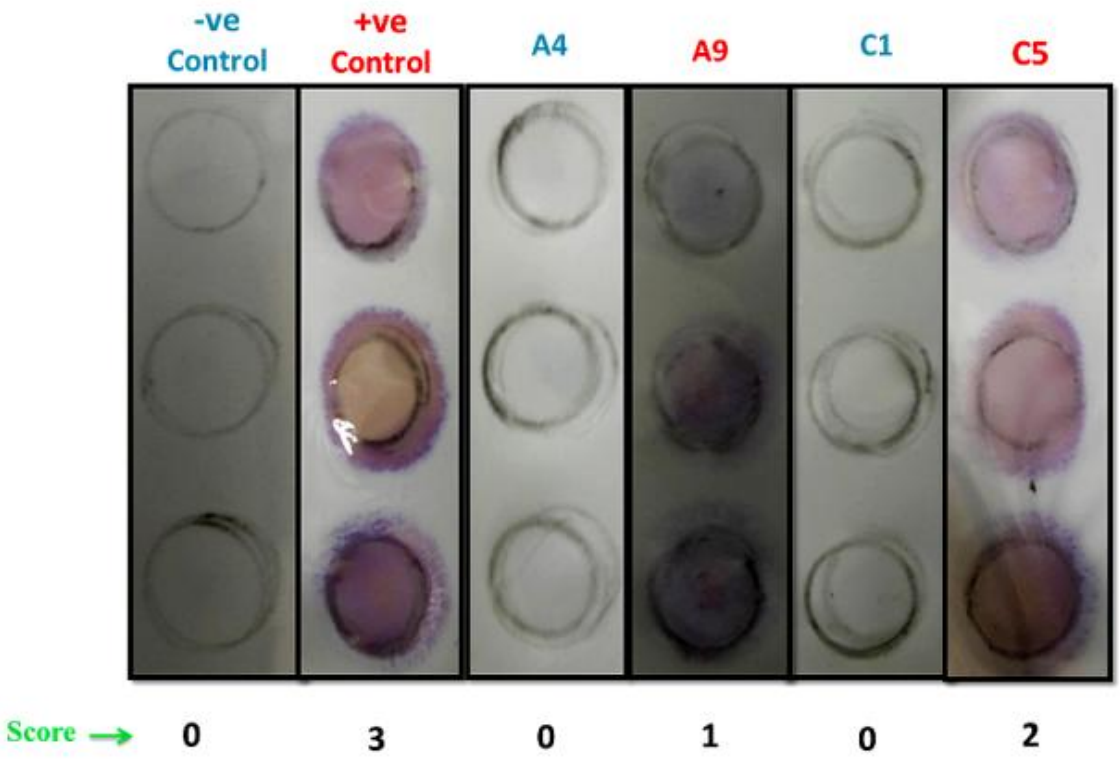

Figure 2.20: Nitrocellulose Membrane Showing the Dot Blot Indirect Immunoassay. Scores: (**0**) No Bioactivity, (**1**) Weak, (**2**) Medium, and (**3**) Strong Bioactivity.

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Appendix VI: Poster Publication

Part of the work presented in this thesis was published as poster in AAPS (American Association of Pharmaceutical Scientists) Annual Meeting and Exposition 2014, *San Diego, California, USA*

Abdul-Ghany E. , Ahmed R., Awad G., Zada S. Improvement of Anti-schistosomal Activity of Praziquantel (PZQ) by Incorporation into Poly (D,L lactide-co-glycolide) (PLGA) Nanoparticles. Poster presentation at the 2014 AAPS Annual Meeting and Exposition; November 2–6, 2014; *San Diego, California*. Poster M1123.

Attached:

1- Published Poster.
2- Accepted Abstract.
3- Certificate of Presentation.
4- Certificate of Attendance.

Improvement of Antischistosomal Activity of Praziquantel by Incorporation into Poly (D,L lactide-co-glycolide) (PLGA) Nanoparticles

M1123

E. R. Abdul-Ghany [1], R. O. Ahmed [2], G. A. Awad [2], S. K. Zada [1]

[1] School of Science and Engineering, American University in Cairo, Cairo, Egypt , [2] Faculty of Pharmacy, Ain Shams University, Cairo, Egypt

Introduction

- *Schistosoma* parasites **(Fig. 1)** infect more than 200 million people in about 74 countries and about 600 million people are at risk. According to WHO, schistosomiasis is the second most prevalent parasitic disease worldwide that has no effective vaccine yet.

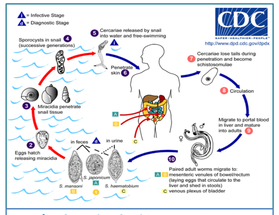


Fig. 1[1]: Life Cycle of *Schistosome* Parasites.

- Praziquantel (PZQ) is the drug of choice for treatment of schistosomiasis since its discovery in 1975. PZQ is not only used for treatment of schistosomiasis, but it is also used for control of other parasitic diseases and it is included on the Model List of Essential Medicines of WHO.
- However, PZQ suffers from several weaknesses. The main one is its rapid and extensive first pass metabolism through the liver resulting in 400-fold less active metabolites. In turn, this necessitates the administration of high oral dose to achieve the therapeutic drug concentration which represents a challenge in drug development. In such scenario, nanoparticulate drug delivery systems have a crucial role to play in improvement of the current anti-parasitic agents.
- **Aim of Work:**

This study aimed at overcoming the inherent drawbacks of PZQ through formulation, optimization and evaluation of PZQ-loaded biodegradable PLGA nanoparticles (NPs) as a preliminary study for design of functional nano-carriers that specifically target and treat schistosomiasis.

Materials and Methods

- **Preparation of PLGA NPs encapsulated with PZQ :** This was done using single (O/W) Emulsion-Solvent Evaporation (ESE)method **(Fig. 2).**

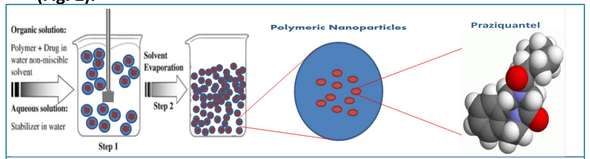
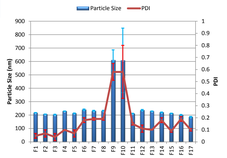


Fig. 2: Schematic representation of polymeric nanoparticles preparation through the high speed homogenization -solvent evaporation method

- **Experimental Design:** various formulation and process parameters were investigated and optimized namely: drug: polymer ratio, surfactant concentration and osmotic effect of adding salts to the external aqueous phase (salt type and salt concentration).
- **Characterization of PZQ-PLGA NPs:**
  - Particle size, Particle size distribution (PDI) and ζ potential using dynamic light scattering method.
  - Drug content of PZQ-loaded NPs and *in-vitro* drug release rate using a validated HPLC method.
  - Surface morphology using scanning electron microscopy (SEM) and Atomic Force Microscopy (AFM).
  - Physicochemical properties: DSC and FTIR
- ***In vitro* parasitological studies** were conducted to compare the schistosomicidal effect of PZQ and PZQ-PLGA NPs on both juvenile and adult *Schistosoma haematobium*. Parasites in different groups were examined after CO_2 incubation for 72 hours in compound RPMI media to observe any ultrastructural changes in their teguments using SEM.

Results and Discussion

a



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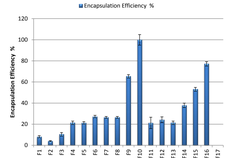
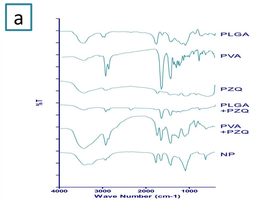


Fig. 3: Characterization of PZQ loaded PLGA NPs prepared by ESE: Effect of different experimental variables.
(a: Particle Size and PDI, b: EE%)%) (F1: PLGA 50:50*, **F2:** PLGA 75:25, **F3, F4 and F5**: 2%, 1% and 0.5% SAA% respectively, **F6 and F7:**5% NaCl and 5% $(NH_4)_2SO_4$ in external aqueous phase respectively, **F8, F9 and F10:** 5%, 10% and 20% $(NH_4)_2SO_4$ in external aqueous phase, **F11 to F15:** Drug to polymer ratio: 1:10, 1:8, 1:6 , 1:2 and 1:1 respectively, **F16****: Medicated optimum formulation prepared after different parameter optimization, **F17:** Blank optimum formulation.)
*PLGA 50:50 was selected for preparation of F3 to F16.
**Selected variables for preparation of F16 were D:P (1:1), SAA% (0.5%), $(NH_4)_2SO_4$ (5%).

- PLGA 50:50 doubled EE% when compared with using PLGA 75:25. **(F1 & F2)**
- Reducing SAA% increased EE%. **(F3, F4 & F5)**
- Addition of salts to the external aqueous phase increased EE%. $(NH_4)_2SO_4$ resulted in more stable emulsion. **(F6 & F7)**
- Lower concentration of $(NH_4)_2SO_4$(5%) increased EE% while keeping the optimized size and PDI unchanged. **(F8, F9 & F10)**
- D:P 1:1 increased the EE% to a great extent. **(F11 to F15)**

a



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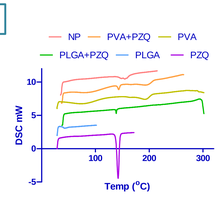
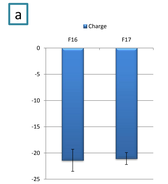


Fig. 5: Physicochemical Properties of Optimized Formula F16 (a) FTIR and (b) DSC

a



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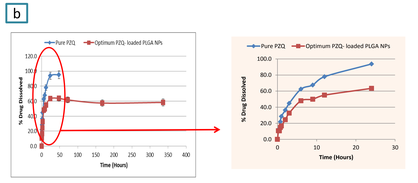

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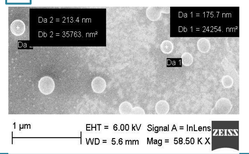

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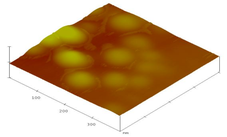

Fig. 4: Characterization of Optimized PZQ loaded PLGA NPs (F16) and Blank PLGA NPs (F17).
(a: c: Charge, b: *In vitro* Release Profile*c: SEM and d: AFM)**
*** *In Vitro* release studies were conducted in phosphate buffered saline (PBS 7.4) at 37°C and 100 rpm. Sink conditions were maintained throughout the study.

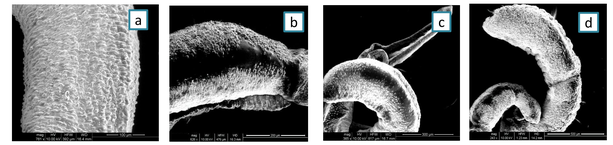

Fig. 6: Scanning Electron Micrographs showing the ultrastructural changes in the teguments of juvenile *S. haematobium*
(a: Negative Control "Untreated", b: Positive Control "Treated with PZQ LC100", c: PLGA NPs , d: PZQ-PLGA NPs)

Conclusion

- Spherical PLGA NPs with a smooth surface were prepared **(Fig. 4c & 4d).**
- The optimized formulation **(F16)** was 200.1±4.68nm in diameter with homogenous size distribution (PDI 0.19±0.02) **(Fig. 3a)** and a negatively charged surface (-21.4±1.1mV) **(Fig. 4a)**
- Both DSC thermogram and FTIR spectrum showed the compatibility between PZQ and PLGA and encapsulation of the drug into the polymer **(Fig. 5a and 5b)**
- PZQ- loaded PLGA NPs showed a sustained release profile from the optimum formulation **(Fig. 4b)** with the highest encapsulation efficiency and drug loading (77.49±2.47% and 38.59±2.47% respectively) **(Fig. 3b)** and a higher schistosomicidal effect **(Fig. 6).**
- PZQ-loaded PLGA NPs could be a potential preliminary delivery system for treatment of Schistosomiasis.

More Information

References:
1. http//www.dpd.cdc.gov

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Improvement of Antischistosomal Activity of Praziquantel (PZQ) by Incorporation into Poly (D,L lactide-co-glycolide) (PLGA) Nanoparticles

E. R. Abdul-Ghany [1], R. O. Ahmed [2], G. A. Awad [2], S. K. Zada [1]
[1] American University in Cairo, [2] Ain Shams University

Purpose

Praziquantel is the drug of choice for treatment of schistosomiasis since its discovery in 1975. According to WHO, schistosomiasis is the second most prevalent parasitic disease worldwide that has no effective vaccine yet. Schistosoma parasites infect more than 200 million people in about 74 countries and about 600 million people are at risk. PZQ is not only used for treatment of schistosomiasis, but it is also used for control of other parasitic diseases and it is included on the Model List of Essential Medicines of WHO. Parasitic diseases, despite their significant global burden, have been rather under researched by drug discovery companies as compared to other diseases mainly due to complexities of parasitic infections and lack of economic motivations. In such scenario, nanoparticulate drug delivery systems have a crucial role to play in improvement of the current anti-parasitic agents. This study aims at overcoming the inherent drawbacks of PZQ through formulation, optimization and evaluation of PZQ-loaded biodegradable PLGA nanoparticles (NPs) for treatment of schistosomiasis.

Methods

PZQ was encapsulated in biodegradable PLGA NPs using single emulsion-solvent evaporation method. Various parameters were investigated and optimized like drug: polymer ratio, surfactant concentration and osmotic effect of adding salts to the external aqueous phase. PZQ-loaded NPs were characterized for drug content and drug release rate using HPLC, particle size, particle size distribution and ζpotential using dynamic light scattering, and surface morphology using scanning electron microscopy. In vitro parasitological studies were conducted to compare the schistosomicidal effect of PZQ and PZQ-PLGA NPs.

Results

Spherical PLGA NPs with smooth surface were prepared. The physicochemical properties of the prepared NPs were investigated and the optimum formulation was selected. The selected formulation was 200.1±4.68nm in diameter with homogenous size distribution (PDI0.19±0.02) and negatively charged surface (-21.4±1.1mV). PZQ- loaded PLGA NPs showed sustained release profile from the optimum formulation with the highest encapsulation efficiency and drug loading (77.49±2.47% and 38.59±2.47% respectively) and higher schistosomicidal effect.

Conclusion

PZQ-loaded PLGA NPs could be a potential preliminary delivery system for treatment of Schistosomiasis.

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