Budesonide Loaded pH-sensitive PLGA Nanoparticles for the Treatment of Inflammatory Bowel Disease

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To my parents and siblings

Abstract

Local drug delivery in contrast to the established systemic drug delivery provides distinctive advances for the safe and effective therapy of inflammatory bowel disease (IBD). The preferred accumulation of small sized nanoparticles in the inflamed gut mucosa explored a new area of targeted drug delivery in the treatment of IBD. The objective of this study was to design a local and targeted nanoparticulate carrier system for the treatment of IBD. For this purpose, budesonide loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles were prepared by oil in water (O/W) emulsion evaporation techniques. Similarly, pH-sensitive nanoparticles were also prepared by coating budesonide loaded PLGA nanoparticles with methyl-methacrylate-copolymer. The prepared uncoated and coated PLGA nanoparticles were characterized for their size, size distribution, shape, drug loading and *in-vitro* drug release profile. The coated nanoparticles showed a differential pH-dependent release profile, while uncoated PLGA nanoparticles showed no effect upon pH change. Furthermore, budesonide loaded uncoated PLGA nanoparticles were first tested for therapeutic efficacy in an inflamed 3D cell culture model, where the drug loaded PLGA nanoparticles significantly affected the secretion of the inflammatory marker IL-8 in comparison to other formulations. After all *in vitro* characterization and stability tests, *in vivo* therapeutic efficacy was assessed using artificially induced colitis animal models. Results from the *in vivo* studies demonstrated the potential of nanoparticles for targeted drug delivery to the inflamed mucosa and moreover this concept can be improved by pH sensitive nanocarriers as observed in this study. However, further experiments are necessary to build up a bridge between research and application to the patient.

Kurzzusammenfasung

Die lokale Wirkstofffreisetzung bietet bei der Behandlung von chronisch entzündlichen Darmerkrankungen (CED) im Hinblick auf die Sicherheit und Effektivität der Therapie deutliche Vorteile im Vergleich zur etablierten systemischen Therapie. Die Akkumulation von Nanopartikeln in der entzündeten Darmschleimhaut eröffnet hierbei neue Anwendungsmöglichkeiten. Das Ziel der vorliegenden Arbeit war die Entwicklung eines nanopartikulären Drug Delivery Systems zur gezielten Behandlung von CED. Mit Budesonid beladene Nanopartikel aus Polylactid co Glycolid (PLGA) wurden mit einer Öl in Wasser Emulsionsmethode hergestellt. Durch das Beschichten von mit budesonid beladenen PLGA Nanopartikeln mit Methacrylsäuremethylestern konnten zudem pH-sensitive Nanopartikel entwickelt werden. Sowohl die nicht beschichteten als auch die beschichteten Nanopartikel wurden hinsichtlich ihrer Größe, Größenverteilung, Morphologie und Wirkstoffbeladung sowie der Wirkstofffreisetzung in-vitro charakterisiert. Bei den beschichteten Nanopartikel wurde, im Gegensatz zu beschichteten Partikel, eine deutliche pH-Abhängigkeit des Freisetzungsprofils festgestellt. Die therapeutische Wirksamkeit von budesonid beladenen, nicht beschichteten PLGA Nanopartikel wurde in einem 3D-Zellkul-turmodell getestet. Im Vergleich zu anderen Formulierungen beeinflussten die PLGA Nanopartikel signifikant die Ausschüttung des inflammatorischen Markers Interleukin-8. Anschließend wurde die therapeutische Wirksamkeit der Partikel auch in vivo in tierischen Colitis-modellen untersucht. Die Ergebnisse dieser in-vivo-Studien zeigten das Potential von Nanopartikeln als Drug Delivery Systeme zur gezielten Wirkstofffreisetzung in der entzündeten Darmmukosa. Darüber hinaus konnte durch die in vivo-Ergebnisse auch gezeigt werden, dass der Einsatz pH-sensitiver Nanopartikel die therapeutische Wirksamkeit weiter erhöht. Um die noch bestehende Lücke zwischen Forschung und tatsächlicher Anwendung am Patienten zu überbrücken, sind jedoch noch weitere Untersuchungen nötig.

List of Abbreviations

AFM	Atomic force microscopy
BSD	Budesonide
C-6	6-cumarin
CD	Crohn's disease
CYP3A4	Cytochrome P450 3 A
DSS	Dextran sodium sulfate
EA	Ethyl acetate
EE	Encapsulation efficiency
FDA	Food and Drug Administration
FITC	fluorescein isothiocyanate
GIT	Gastrointestinal tract
HPLC	High performance liquid chromatography
IBD	Inflammatory bowel disease
IFN-γ	interferon gamma
IL	Interleukin
IL-1β	Interleukin-1 beta
MPO	Monoamine peroxidase
MQ	Milli-Q
MS	Microspheres
NaOH	Sodium hydroxide
NOD2	nucleotide-binding oligomerization domain-containing protein 2
NP	Nanoparticles
NS	nanospheres

O/W	Oil in water
PBS	Phosphate buffer saline
PCS	Photon correlation spectroscopy
PLA	Poly (lactic acid)
PLGA	Poly (lactic acid-co-glycolic acid)
PVA	Polyvinyl alcohol
SD	Standard deviation
SEM	Scanning electron microscopy
SGF	Simulated gastric fluid
SGIF	Simulated gastro-intestinal fluid
TEM	Transmission electron microscopy
Th1	T helper cells 1
Th2	T helper cells 2
TNBS	Trinitrobenzenesulfonic acid
TNF-α	Tumor Necrosis Factor alpha
UC	Ulcerative colitis
W/W	Weight by weight

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Chapter 1

Introduction

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1.1 Background and literature overview

Inflammatory bowel disease (IBD) comprises many chronic idiopathic inflammatory conditions of the gastrointestinal tract with Crohn's disease (CD) and ulcerative colitis (UC) being the most prevalent and commonly studied [1, 2]. Although IBD has been extensively investigated for more than half a century, the pathogenesis still remains unknown [3]. UC and CD present similar symptoms for example diarrhea, bloody stool, weight loss, abdominal pain, fatigue and fever. However, pathological lesions and clinical manifestation can distinguish CD from UC. In UC inflammation mainly affects the innermost mucosa without involvement of deeper tissues like muscularis or serosa and is confined to the colon and rectum. In the case of CD, inflammation is transmural i.e. affecting the entire wall of the intestine down to the serosal layer, and can occur over the entire length of the large and small intestine, sometimes even involving the mouth [4, 5].

The pathogenesis for CD and UC is to some level understood. IBD is believed to be caused by an exuberant continuous inflammatory response to commensal intestinal bacteria in a genetically susceptible host. Generally the risk factors which are most firmly established for the development of IBD are related to genetics, for example mutations in NOD2 encoding genes. The risk of CD in a sibling of a CD patient is significantly higher, and approximately 15 % of the patients with IBD have a first degree relative with the disease; the pattern of inheritance is however not clear [6, 7]. In addition, several environmental risk factors seem to contribute to the pathogenesis, but are less well understood and difficult to tackle. Possibly relevant environmental factors include prenatal events, breastfeeding, childhood infections, microbes, smoking, use of oral contraceptives, diet, hygiene, occupation, pollution and stress [8, 9]. Incidence rates of IBD are highest in North America, the UK and northern Europe, but have stabilized in these areas in recent years. An increase in incident rates has however been reported in previously low incidence areas such as developing countries; Asia and southern Europe, hinting again at life style associated risk factors. The highest prevalence rates have been recorded for white individuals (43.6 per 100 000), considerably higher than in Asian peoples (5.6 per 100 000) or Blacks (4.1 per 100 000) [10, 11].

As mentioned, IBD is thought to result from an over-reaction of the intestinal immune system to the indigenous intestinal flora and other luminal antigens. Multiple complex path-

ways might be involved in the deregulation of the inflammatory cascade, as evidenced by *in vivo* investigations in human IBD patients and transgenic animal models of colitis [12]. In IBD lowered epithelial resistance and increased permeability of the inflamed and non-inflamed mucosa have been observed and different mechanisms for the increased permeability ranging from T-cell mediated disruption of tight junction proteins to enteric neuron dysfunction have been proposed [13–15]. Moreover, an increased level of interleukin 12 (IL-12) cytokine has been observed in a patients suffering from CD which leads to increased T helper cells (Th1) responses and interferon γ (IFN-γ) release. The overbearing activation of Th1 cells and macrophages produces uncontrolled inflammation and activation of metalloproteinases which then cause tissues damage [16, 17]. Furthermore, resistance of activated T cells to normal apoptosis, leading to continuous inflammatory cycles, has been proposed as a key step in the pathogenesis of CD [18].

1.1.1 Drug molecules for IBD therapy

Traditionally, the aim of IBD therapy has been to ameliorate the symptoms of active disease and to prolong remission phases. Treatment of IBD depends on different factors relating to both drug and patient. Drug related characteristics include pharmacodynamics and pharmacokinetics while patient-oriented factors are mainly dependent on the severity of disease, location of inflammation and response to initial therapy. As a permanent cure is not available to date, patients depend on life-long application of drugs [19].

Nowadays, a wide arsenal of drugs such as 5-aminosalicylates, corticosteroids, immunosuppressives and tumor necrosis factor alpha (TNF-α) antibodies are available for IBD therapy applied in a multi-tiered approach. Still, at one point of their life 70-80% of patients with CD and 25-40% of UC patients have to undergo abdominal surgeries (in most cases resections) due to failure of other therapeutic options [20]. Furthermore, severe adverse effects of long term medication negatively affect the quality of life of IBD patients demonstrating the need for better targeted formulations.

As induction and maintenance of remission are the main objectives in IBD therapy therefore 5-aminosalicylic acid (5-ASA, mesalazine) is recommended as a first line therapy for patients with mild to moderate UC, being in practice as oral and rectal formulations. Furthermore, pro-drugs such as sulfasalazine (5-ASA linked to sulfapyridine), balsalazide (5-ASA linked to

4-aminobenzoyl-β-alanine) or the 5-ASA dimer olsalazine are available [21, 22]. No difference in 5-ASA pharmacokinetics and systemic exposure were found on comparing an orally administered mesalazine formulation to a mesalazine pro-drug [23]. Aminosalicylates act by inhibiting the cyclooxygenase and 5 lipoxygenase pathways of arachidonic acid metabolism, and can also modulate immune responses [24]. Sulfasalazine has shown great benefits for induction of remission at doses of 3000-4500 mg per day in patients with active CD, but due to sulfapyridine-related intolerance in some patients, the use of sulfasalazine is limited [25, 26]. In patients who do not respond to 5-ASA or prednisone, other systemic glucocorticoids are applied [27]. Corticosteroids have an effect on both immune and inflammatory responses, mainly by inhibiting pro-inflammatory cytokines [28].

In a population based study of IBD patients treated with corticosteroids, 58% of patients with CD showed complete remission after one month of treatment, 26% showed partial remission and in 16% patients no remission was achieved. In UC patients, 54% showed complete remission in the same period, 30% showed partial remission and 16% illustrated no response to therapy. However, after one year outcomes statistics revealed that 28% of patients with CD and 49% of patients with UC had a prolonged response, 32% of patients with CD and 22% with UC had become corticosteroid dependent and 38% of patients with CD and 22% of patients with UC needed surgery [29]. These epidemiological based findings illustrate that, although corticosteroids remain a first line therapy in active IBD treatment, corticoid dependency and acquired corticoid resistance still present common problems [30]. Such findings therefore accentuate the need for steroid-sparing medication in IBD.

Locally acting glucocorticoids, such as budesonide, can help to reduce the rate of adverse events but their use does not overcome the issue of corticoid resistance. Budesonide has a strong affinity for the corticosteroid receptors, but a very low systemic bioavailability of only 10% to 15% after oral administration due to extensive first pass metabolism in the liver by cytochrome P-450 enzymes [31]. In one targeted delivery study where budesonide was designed and formulated as a pH and time dependent dosage form for ileum and colon delivery, a distinct decrease in systemic corticosteroid side effects was noted [32]. Furthermore, it was hypothesized in a controlled trial that budesonide 9 mg per day is better in efficacy than placebo and more effective than an oral daily dose of 4000 mg of 5-ASA for the induction of remission in active disease.

In patients with severe IBD and in those who do not respond to corticosteroids, treatment with immunosuppressive agents such as cyclosporine or methotrexate is the next line of therapy. Patients who receive oral 5-ASA compounds, cyclosporine, or tacrolimus or those who are steroid dependent can also be treated with azathioprine and mercaptopurine. However, the safety and efficacy data of azathioprine and mercaptopurine for the therapy of UC are conflicting [33].

Advances in the immunological study of IBD and efforts in the field of bioengineering have led to new therapeutic strategies targeting main features of the inflammatory process [34]. TNF-α is a mediator of inflammation [35, 36] and an end-stage object of the inflammatory cytokine cascade. It induces inflammation and is involved in many systemic and cutaneous inflammatory diseases [37]. TNF-α production in the intestinal mucosa of IBD patients is considered to play a major role in the initiation and proliferation of the disease. Thus, TNF-neutralization has been recommended as a target therapeutic intervention in various inflammatory diseases including arthritis and IBD [38–41].

The human chimeric monoclonal antibody to TNF-α, infliximab, previously known as cA2, is a genetically constructed IgG1 murine antibody which binds both the soluble sub unit and the membrane-bound precursor of TNF-α [42, 43]. Infliximab is now used for the induction and maintenance therapy of CD and UC [44–47]. Its mechanism of action is to block biological activities of TNF-α, acting as an agonist, apparently inhibiting TNF-α interaction with its receptor [48]. In addition to infliximab, two other biological agents, adalimumab and certolizumab, that also inhibit TNF-α, have been approved by the FDA for IBD therapy.

Certolizumab pegol is a pegylated, Fab´fragment of a humanized monoclonal antibody and has high binding affinity for TNF-α [49]. The pegylation enhances the stability and increases half-life thus allowing for a reduction in the dose and also resulting in a decreased antigenicity [50]. In patients with moderate to severe CD, certolizumab pegol showed effective induction of remission [51], with better efficacy compared to other monoclonal antibodies such as infliximab and adalimumab. Certolizumab does not contain an Fc portion and therefore does not induce antibody cellular cytotoxicity or apoptosis [52]. In a clinical study induction and maintenance of remission was achieved with certolizumab pegol therapy, which was associated with modest improvement in response rates but no remarkable progress in remission rates [49].

The biological based therapies mentioned above are all applied intravenously or subcutaneously, and as such all face issues with stability/half-life and risks of infections due to unspecific, systemic immune effects. Instead, local therapy would be beneficial, but targeting to the inflamed intestinal mucosa and stability in the GI environment has to be assured.

1.1.2 Drug delivery strategies for IBD therapy

In inflammatory bowel diseases, disease severity, pattern and location of disease within the gastrointestinal tract are important treatment parameters [21, 22, 53]. Rectal formulations such as suppositories, foams and enemas have been efficiently applied in UC when it occurs in lower parts of the colon. However, rectally administered dosage forms are not effective in case of a pancolitis, where inflammation occurs in the ascending or transverse colon areas. The oral route has been considered to be an effective route of delivery for various formulations in the treatment of CD, which can affect any part of the GIT from mouth to anus. Still, limitations exist, in particular systemic absorption from the gut and small intestine and extensive first pass metabolism of active pharmaceutical ingredients, leading to only a fraction of the active compound reaching the actually inflamed areas [24]. Thus, treatment for IBD needs a carrier system that could deliver the therapeutic agent exclusively to the target site to avoid systemic absorption and reduce adverse effects. An increase in local drug concentration would also result in enhanced therapeutic activity and a reduction in dose and frequency of drug application.

Colon targeted delivery systems are an established tool for site directed delivery of various therapeutic agents in the treatment of IBD but the pathophysiological changes observed in the inflamed intestine in CD and UC can limit the reliability and effectiveness of these targeting strategies.

Prodrugs cleaved and thus activated by colonic enzymes have been successfully evaluated in the treatment of IBD. The most common example in this context is sulfasalazine with its active compound 5-ASA showing a cleavage rate into sulfonamide and mesalazine of about 75% [54, 55]. In general, the prodrug strategy can be varied by using amino acid conjugates, glycoside conjugates, azo conjugates, glucoronide and sulphate conjugates [56]. The main risk factor of this drug delivery approach is its dependency on the enzymatic activity of the colonic microflora. In the case of UC, the bacterial population is mostly unchanged, but in CD

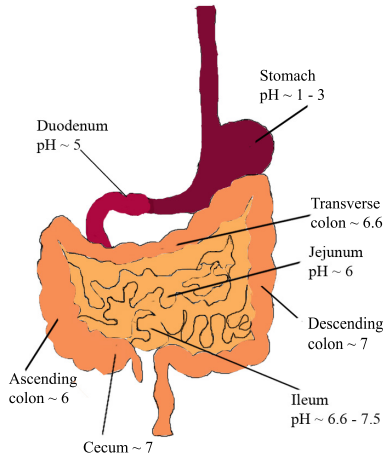

Figure 1.1: Schematic presentation of the gastrointestinal tract illustrating pH values at different areas of the gut.

variations in enzymatic activity have been observed, leading to uncertainty as to the reproducibility of this therapy [57].

Furthermore, pH dependent drug release systems have been broadly applied which withstand lower pH and release the drug only at the weakly acidic to neutral pH of the distal ileum and colon (pH ≥ 6; Figure 1.1). Some pH controlled formulations have been marketed in Europe and the US, but still a controversy exists about the reliability of the targeting method. These formulations were developed for a healthy human gut, while patients with IBD often show lower colonic luminal pH ranging from 5-7, but sometimes dropping as low as pH 2.3, 2.9 or 3.4 risking incomplete drug release and reduced efficacy of the formulation [58–60].

Apart from the aforementioned challenges to the current therapy another important factor is diarrhea, which is a major symptom of IBD [61] , minimizing the residence time of the therapeutic agent at the site of action and thus broadly reducing the efficacy of the available medications. The currently available drug delivery system such as enteric coated capsules, tablets, granules or pellets are less efficient because of the high frequency of diarrhea (66% – 92%) associated with IBD [62], therefore an alternative delivery strategy is needed.

1.2 Nanomedicine and IBD

There are many definitions of the terms “nanobiotechnology”, “nanomedicine” and “nanomaterial” according to different scientific disciplines. Unfortunately there is no clear or meaningful definition available of nanomedicine either in international conferences, journals, articles, text books or unpublished talks. After careful consideration in a consensus conference, the European Science Foundation’s (ESF) Forward Look Nanomedicine defined nanomedicine as the science and technology of diagnosis, treatment and prevention of disease, injury, pain and the improvement of human health [63]. The aim of nanomedicine is further defined as using engineered devices and nanostructure at a molecular level for the control, repair, defense and improvement of all human biological systems. Worldwide debates are however ongoing to specifically and exclusively define what really constitutes “nanomaterial” for the purposes of safety regulation. The European Commission (EC) Joint Research Centre Report [64] uses a working definition for the nanoscale as being approximately 1 to 100 nm; any material in this dimension is then considered nanomaterial. Moreover, the term nanotechnology has been differentiated from nanomedicine. Nanotechnology is the field of science and technology of objects dominated by surface atoms whereas nanomedicine is a branch of science of delivering drugs or biochemicals to specific cell types through endocytosis either in the form of nano-sized drug particles or nano sized carriers, typically in a size range between 50 nm and several hundreds of nanometers [65].

Nanomedicine (NM) i.e. using nanotechnology for medical purposes has generated much excitement and promise for the development of novel therapeutics and diagnostics [66]. The potential of nanomedicines for active or passive targeting to diseased areas, to protect cargo molecules from degradation, and to control drug release over time (thus decreasing dosing frequency) is of course very attractive. This holds also for oral drug delivery, where especially in the context of inflammatory bowel diseases an increasing interest is recently observed [67–71]. Similarly as in cancer therapy, targeting the sites of inflammation and formation of local drug depots may enhance and prolong the intended pharmacological effects, while at the same time reduce systemic adverse effects. Besides small molecules, the protection against the harsh environment of the gastrointestinal tract allows local delivery of fragile biologicals, opening up the oral route for new compounds and also new therapeutic targets. This review

discusses various approaches for the drug therapy of IBD, focusing on the potential of NM and the steps still needed to be taken towards translation into the clinic.

NMs are used as carriers, having the ability to incorporate both hydrophilic and hydrophobic drugs and to be administered to the body by various routes [72]. NMs have the ability to overcome biological barriers and protect their cargo against degradation, and are therefore able to effectively deliver their passenger compound to the site of action. NMs have been described to passively accumulate in diseased regions of the body by virtue of their size alone e.g. in tumor tissue or sites of infection and inflammation due to changes in vascular permeability and retention [73]. Moreover, active ligand and receptor mediated targeting can be utilized to allow for an increased accumulation of NMs at the site of action.

In NMs carrier material, particle size and surface charge play a key role as such physical properties determine cellular uptake and interaction with biomolecules. Another important aspect of NMs is their high surface area-to-volume ratio. Small particles possess more interaction sites as compared to large size particles which may help to regulate pharmacokinetics of drug release [74]. Nanoparticles can be either colloidal dispersions of nanocrystalline drug, solid excipient particles in which the drug is dispersed, adsorbed or dissolved in the matrix, or nanocapsules in which the drug is confined to an aqueous or oily core surrounded by a shell like wall [75]. NMs are preferably made from biocompatible and biodegradable polymers, of either natural origin, e.g. gelatin and albumin, or synthetic origin, e.g. poly lactide (PLA), poly glycolide (PGA) and their co-polymer poly lactide-co-glycolide (PLGA). Drug release from such nanoparticles occurs either by diffusion, swelling, erosion or degradation in the body. Various approaches have been reported for the preparation of nanoparticles such as emulsion evaporation/diffusion, nanoprecipitation, or salting-out techniques [76].

The treatment options and strategies available for IBD prior to the last decade were not efficient and their use is now limited due to the adverse effects which are mainly related to the lack of targeted delivery [77] and the inconsistent nature of IBD which varies greatly from patient to patient. NMs present new possibilities to overcome these limitations. By passive targeting alone, it is possible to facilitate the accumulation of drug loaded nanocarriers in the inflamed intestinal areas. Lamprecht et al [78] initiated the interaction of micro- and nanoparticles with the inflamed mucosa as a targeting principle in IBD. A size dependent deposition of fluorescently labeled polystyrene particles was observed in a trinitrobenzenesulfonic acid

(TNBS) rat model of colitis. Particles were found in abundance at the thicker mucus layer adjacent to the inflamed tissues and in far lower quantities in the healthy tissue. Furthermore, the study revealed an inverse relationship between particles size and adherence of particles to the inflamed intestinal mucosa: particles of approximately 100 nm in size exhibited stronger adherence to the inflamed area compared to 1µm and 10 µm sized particles.

Interaction with the intestinal mucus, which is significantly increased in production in the case of IBD [79] can account for some of the selectivity observed. In addition, immune cells such as macrophages and T-cells play an important role in the pathogenesis of IBD and strongly invade the inflamed tissue. In particular antigen presenting cells such as macrophages and dendritic cells actively engulf particulate carrier systems by endocytosis [80, 81]. Microspheres prepared from biodegradable polymers and copolymers such as polylactic acid and polyglycolic acid [82, 83] and various starches and cross linked starches [84, 85] are observed to be successfully internalized by macrophages, however the exact mechanism of phagocytosis is still not clearly understood [86]. Still, uptake by immune cells might significantly contribute to the drug carrier accumulation in the inflamed intestine and also provide a successful strategy to target key players in the inflammation process.

1.2.1 Nanomedicines for small molecule drugs

Rolipram, a phosphodiesterase IV inhibitor, was loaded into PLGA nanoparticles and was tried as a first model drug to passively target the inflamed intestine in a study conducted by Lamprecht et al. [87]. In this study, drug loaded particles were prepared by the emulsion evaporation method with a high encapsulation efficiency of approximately 85 %. *In vitro* drug release results showed a biphasic release profile with an initial burst release of up to 40% followed by slow and sustained release for 7 days. *In vivo* testing was carried out in Wistar rats suffering from TNBS colitis. After oral application of rolipram loaded PLGA nanoparticles clinical activity score and myeloperoxidase (MPO) activity were significantly reduced, as was the case for animals receiving free rolipram solution. However, animals which were on rolipram solution displayed a strong relapse within 5 days after treatment was stopped, while animals treated with nanoparticles showed a continuous reduction in inflammation level. Furthermore, the rate of central nervous system adverse effects usually associated with oral rolipram treatment was significantly reduced further supporting the depot formation and passive targeting effect

of rolipram PLGA nanoparticles (NP).

In addition to PLGA, polycaprolactone (PCL) can be used as matrix material for rolipram loaded nanoparticles [78]. While encapsulation efficiency is comparable for both biodegradable polymers, *in vitro* drug release in simulated intestinal fluid containing pancreatin is faster from PCL NP than from PLGA NP. Rolipram PCL NP released 80% of the drug after 48 hours while PLGA NP released only 50 % after 48 hours and 80% after 7 days. A correlation to *in vivo* efficacy has not been attempted so far, but the residence time of 3-5 days should favor the PLGA rolipram formulation. Nanoparticle targeting to the inflamed intestine can not only be applied to experimental drugs such as rolipram, but can also improve pharmacokinetics of traditional IBD therapeutics where state-of-the-art delivery devices fail. As an example, 5-ASA loaded PLGA nanoparticles were prepared by a modified emulsion diffusion and nanoprecipitation method [88]. Particles ranged from 135 to 210 nm in size but entrapment efficiency of hydrophilic 5-ASA was low at 1.5 to 2.5%. Particles were administered once a day either orally or rectally to male Wistar rats suffering from acetic acid induced colitis. While nanoparticle therapy improved the clinical disease activity score and maintained the anti-inflammatory effect, the relevance of the results has to be questioned, as no comparison to commercial 5-ASA formulations was conducted (only the free drug solution was used as a control). The animal model used is also less well established than the DSS or TNBS colitis models, limiting comparability.

Several strategies have been investigated to limit premature drug release from nanoparticles in the upper GI thereby reducing the risk of drug degradation and systemic absorption. As for traditional macroscopic formulations, the use of pH sensitive polymer coatings (e.g. polymethacrylates) is a popular approach. In this regards tacrolimus (FK506) loaded PLGA nanoparticles were prepared and entrapped within pH sensitive Eudragit P-4135F microspheres [89]. *In vitro* release profiles of this formulation demonstrated strongly pH dependent kinetics, with drug release only occurring at pH ≥ 7.4. In-vivo testing of therapeutic efficacy in a TNBS rat colitis model however showed only limited anti-inflammatory potential of the formulation, as only some disease parameters (myeloperoxidase activity and colon/body weight) were significantly different to those of the non-treated control. Still the nanoparticle in microparticles system was superior to free tacrolimus solution, to non-encapsulated PLGA tacrolimus nanoparticles and to plain tacrolimus loaded Eudragit® S100 microspheres [90].

The overall poor performance of the nanoparticle in microparticles carrier system may be associated with the preparation method and the high drug solubility in the organic solvents used for PLGA particle preparation and subsequent coating to microparticles. In agreement with this hypothesis, reduced encapsulation efficiency and accelerated *in vitro* release have been reported for Eudragit® S100 coated PLGA microparticles [32].

In contrast to coating with Eudragit® S100, Makhlof et al. [91] blended PLGA and the pH sensitive polymer (Eudragit® S100) in a 1:1 and 2:1 W/W mixture of PLGA and Eudragit® S100, was co-precipitated to entrap budesonide as a model drug. The 1:1 ratio formulation demonstrated good entrapment efficiency of greater than 85% and reduction in drug release was comparatively good at pH 1.2 and pH 6.8 to 20 to 28%, respectively. Only at pH 7.4 the total residual drug amount was released, within a 24 hour period. The enhanced degradation of chitosan particles by colonic enterobacteria is also a useful strategy to target drug release to the distal part of the intestine. In this regard, Crcarevska et al [92] described a one-step spray drying process to formulate budesonide loaded chitosan-Ca-alginate microparticles coated with Eudragit® S100. Particles coated with Eudragit® S100 showed a very low release at pH 2 and 6.8. Instead 60% of drug release occurred at pH 7.4 after 24 hours. For *in vivo* testing the formulations were administered by oral gavage at a dose of 167 µg/kg/day for 5 consecutive days in the form of a 1 ml suspension. Clinical activity score and colon body weight ratio were evaluated. Their results showed significant reduction in colitis severity after application of particles coated with Eudragit® S100 compared to both particles without coating and to budesonide alone.

Apart from the pH sensitive nanospheres, another approach to retard the initial burst release in the upper part of GI system was proposed by Pertuit et al [93]. 5-ASA was covalently coupled to PCL and particles were prepared by an emulsion solvent evaporation or nanoprecipitation method. Particles ranged in size from 200 to 330 nm. The *in vitro* release profile showed a low burst release of 20% in PBS pH 6.8 in the first hour which was associated to non-coupled 5-ASA or to immediate hydrolytic cleavage of 5-ASA-PCL on the particle surface. Therapeutic activity was studied in the TNBS colitis model. Clinical activity score and MPO activity revealed that 5-ASA NP administered orally at a dose of 0.5 mg/kg body weight showed less efficiency compared to 5-ASA solution 100 mg/kg but was comparable to 30 mg/kg drug solution, giving an approximately 60 fold increase in therapeutic efficacy.

In contrast to coupling of drug and polymer followed by particle preparation from the modified material, particles may also be pre-formed with the drug then being covalently linked to the surface. Moulari et al [94] attached 5-ASA to the surface of porous silica nanoparticles by a four step reaction process using a succinimide anhydride linker. Final particle size was approximately 140 nm with a high loading rate of 151 ± 62 mg 5-ASA per 1000 mg of silica NP, and particles had a negative zeta-potential. The *in vitro* drug release profile was studied in PBS and simulated intestinal fluid at pH 6.8. Results showed almost no release in the first 5-6 hours of release profile study in both PBS and simulated intestinal fluid. An 80% drug release was achieved after 48 hours in simulated intestinal fluid due to cleavage of the succinimide bridge by enzymes such as pancreatin, while no drug release was noted in PBS after 48 hours. The release of Me5ASA after a period of 6 to 8 hours, may be explained by the high degree of surface modification and resulting steric hindrance of enzyme access to the cleavage site. It also may allow for a colon delivery of a maximum drug dose and help to avoid 5-ASA absorption in the upper GIT.

In addition to the aforementioned nanoparticle-based strategies, the use of liposomes, which may also be considered as nanomedicines, have also been applied to IBD treatment. Because of stability issues in the harsh environment of the GI tract liposomes have mostly been applied parenterally. However, Jubeh et al [95] compared the adhesion of differently charged liposomes to inflamed intestinal tissue vs. healthy tissue, and from this work the authors concluded that anionic liposomes are useful for the topical delivery of anti-inflammatory drugs in IBD therapy due to their high adherence to the inflamed tissue as compared to neutral or cationic liposomes. The same authors [96] reported a strategy for the local delivery of anti-oxidants in IBD which would circumvent issues related to the stability of liposomes, thus encouraging the use of such carriers. In this work, liposomes were loaded with superoxide dismutase, tempamine and catalase and applied rectally via enemas two times a day for three consecutive days. The MPO activity, thiobarbituric acid reactive species (TBARS) amounts and colon weight were evaluated, and it was reported that negatively charged liposomes mitigated the BNDS (dinitrobenzene sulfonic acid) induced colitis. Another liposomal strategy was reported by Kesisoglou et al [97]. In this study, 5-ASA was encapsulated in non-phospholipid liposomes, while 6-mercaptopurine (6-MP) was encapsulated into phospholipid liposomes. Both were administered intraluminally to healthy rats and to rats with colitis. 5-ASA loaded

non-phospholipid liposomes showed a reduced drug bioavailability while 6-MP loaded phospholipid liposomes failed to improve local delivery.

1.2.2 Delivery of next generation IBD therapeutics

In addition to established small molecule drugs, nanoparticulate drug delivery systems also have the ability to incorporate biologicals, the next generation of IBD therapeutics which so far for stability reasons have been restricted to intravenous application. Encapsulation into nanoparticles provides not only targeting to the site of the inflammation but also affords protection from the harsh gastric and intestinal environment and resolves issues related to the half life time of labile biologicals in the systemic circulation.

Low molecular-weight heparin (LMWH) has been shown to be efficient for the therapy of IBD when administered parentally [98, 99], but due to hemorrhagic adverse effects [100], its use as a typical therapeutic option in the pharmacotherapy of IBD has been prevented. To reduce this risk, Yann et al [101] proposed a local delivery strategy for LMWH to locally target the inflamed intestinal mucosa - as heparin has a minimal tendency to cross the intestinal mucosa, this would ultimately reduce adverse effects. In their study, heparin was loaded into pH sensitive microspheres prepared from Eudragit p-4135 by double emulsion techniques. To determine inflammation, colon body weight, alkaline phosphatase and myeloperoxidase activity were assessed. Orally delivered LMWH microspheres have shown promising results and mitigated the colitis to the same extent as rectally administered LMWH solution when myeloperoxidase activity was quantified, moreover the orally delivered microspheres and the rectally administered solution was observed to be superior to the subcutaneously administered LMWH solution. The bioavailability studies of LMWH from orally delivered LMWH microspheres revealed a notably low systemic availability i.e. less than 5% availability of LMWH indicating a low potential for adverse effects.

Laroui et al [102] described a nanoparticulate system for delivery of the anti-inflammatory tripeptide Lys-Pro-Val (KPV) which was loaded alongside the stabilizer protein BSA into (PLA) nanospheres by a double emulsion evaporation method. To avoid premature release from the biodegradable polymeric carrier and subsequent degradation after oral administration the formulation was orally co-admini-stered to mice with a chitosan-alginate hydrogel. In situ in the mouse stomach hydrogel beads are formed protecting the nanoparticles. *In vitro*

studies at pH 1-3 showed no swelling or collapse of the beads ensuring stability during gastric passage. Release of cargo started at pH 5 which can facilitate nanoparticle delivery to the colon. An *in vivo* efficacy study in a DSS colitis model showed enormous benefits resulting from oral KPV nanoparticle application. Not only was the drug intact after gastric passage, but mucosal targeted delivery by daily gavage for 7 days increased anti-inflammatory activity by a factor of 15,000 compared to intravenous (iv) application as quantified by histological parameters and TNF-α and IL-1β cytokine levels.

Furthermore, the nanoparticulate delivery approach is not only restricted to peptides and proteins but may also be applied to (siRNA) and other nucleotide drugs. Accordingly, TNF-α-siRNA was loaded into polylactide nanoparticles [103] of approximately 380 nm size and *in vitro* anti-inflammatory effect and TNF-α silencing was confirmed on pre-inflamed mouse macrophages. An *in vivo* study was conducted in a DSS colitis model and to ensure stability during gastric passage the nanoparticles were co-applied with in situ forming hydrogel beads as reported previously [102]. Indeed, tissue TNF-α levels were statistically significantly reduced in mice treated with nanoparticle formulation. As another similar approach for the orally delivery of siRNA, to down regulate the expression of TNF-α, the siRNA was encapsulate into type B gelatin nanoparticles which were then further entrapped in poly (epsilon caprolactone) (PCL) microspheres, in order to form nanoparticles-in-microsphere [104]. The therapeutic efficacy of this carrier system was evaluated using a DSS-induced acute colitis model following oral administration of the nanoparticles-in-microsphere delivery system. Gene silencing led to down regulation of the level of TNF-α, and also suppressed the expression of IL-1β, INF-γ and chemokines (MCP-1). A reduction in myeloperoxidase activity and an increase in body weight were also noted, suggesting the clinical potential of a TNF-α loaded nanoparticles-in-microsphere delivery system.

A rather sophisticated system for the delivery of biologics described so far is based on thioketal nanoparticles (TKN) [105]. The novel polymer poly-(1-4 phenylenacetone dimethylene thioketal) is sensitive to reactive oxygen species (ROS) but resistant to acid-base-and protease-catalysed degradation. After loading with anti-TNF-α siRNA (by a single O/W emulsion method), the nanoparticles of approximately 600 nm in size were seen to accumulate in the inflamed intestinal tissue and also were efficiently taken up by macrophages. In inflammation, the activated immune cells release ROS as a mediator; the thioketal is sensitive

to ROS, meaning that the thioketal nanoparticles only release their cargo upon interaction with ROS. Furthermore, orally applied siRNA encapsulated in β1,3-D-glucan particles [106] was evaluated in IBD therapy, aiming to silence Map4k4, a key mediator of the inflammatory cascade. Results showed potent gene silencing in mouse macrophages both *in vitro* and *in vivo* at a dose of 20µg/kg, so creating a new strategy for siRNA oral delivery.

1.3 From mouse to men - translation into the clinic

A variety of IBD animal models have been used as experimental tools to better understand disease pathogenesis, to investigate the complex interactions that may contribute to the disease and to follow up on appropriate treatment strategies. The available IBD animal models comprise chemically induced models, adoptive transfer models and genetically modified animals [107].

Chemical agents which have been used to induce IBD in animal models include acetic acid [108], DSS [109, 110], carrageenan [111], TNBS [112, 113] and very recently sodium hydroxide [114], with the DSS and TNBS models being the most commonly used. Both agents cause destruction of the intestinal barrier. Inflammation induced by TNBS, a haptenating substance, is more chronic due to an increase in production of IL-12 and IL-17, while the inflammation induced by DSS is acute, with a prominent IL-4 and IL-10 cytokine response and a simultaneously observed decrease in TNF-α, IL-6 and IL-17 [115]. Repeated cycles of low dose DSS application can shift the immune response to a more chronic phenotype. Regardless, epithelial damage is the primary feature of both the DSS and TNBS models. In contrast, adoptive transfer models are based on transferring T cells or other immune cells from one mouse into a histocompatible host. Colitis arises by disruption of T cell homeostasis. The adoptive transfer model is responsive to a variety of treatment options and has proven immensely helpful in understanding the T cell contribution to the pathogenesis of IBD [116].

The most relevant IBD models are the genetically modified animals, as genetics are thought to have a considerable role in the disease pathogenesis in humans. In most transgenic animal models key genes encoding cytokines related to immune homeostasis (IL2, IL-10 and TGF β) or signal transduction (STAT-4tg, IκBα) are knocked out or altered in expression. In contrast, only few epithelial models can be found, such as mdr 1-deficient mice or N cadherin dominant

negative mice [117]. In particular, IL-10 knock-out mice have been extensively studied as the *in vivo* pathogenesis in such mice closely mimics human IBD, in particular with regards to the role of T-cells and intestinal flora. In the pharmaceutical context, the IL-10 knock out model is particularly valuable for the evaluation of IL-10 plasmid and protein delivery. The establishment of more clearly defined clinical phenotypes and application of genetic tests in clinical medicine would enable more accurate matching of mouse models to patients, which will eventually lead to more appropriate therapy. In terms of monitoring the effectiveness of therapy and classification, the identification of appropriate diagnostic biomarkers would be of important benefit in this context [107].

Clearly, at this point in time, none of the available animal models represents the exact pattern and complexity of the disease found in human IBD. Instead, they have to be considered a useful tool studying pathophysiological mechanisms and evaluating experimental therapeutic strategies such as nanomedicine [118]. Testing of novel drugs and formulations in more than one animal model can strengthen the validity of the findings, as can the combination with complex *in vitro* models comprised of human intestinal epithelial cells and immune cells [119, 120].

In addition to variations in disease pathogenesis, simple physiological species difference might impede the transfer of animal findings into men, especially with regards to different targeting strategies [121]. pH dependent targeting is difficult to evaluate in mice, as small and large intestinal pH is significantly lower than in humans, e.g. looking at ileal pH which is 4-4.5 in mice vs. pH 6-6.8 in men [122].

Furthermore, dimensioning has to be considered both with regards to total intestinal length as well as mucosal thickness. Clearly illustrating the impact of these physiological parameters, Schmidt et al. [123] recently found an accumulation of microscale placebo PLGA particles of around 2 µm in diameter in ulcerated rectal tissue of human IBD patients, while no accumulation of 200 nm nanoparticles were observed. However nanoparticles were detected in traces in the mucosa of these patients. Similarly, in another recent finding by Lautenschläger et al. [124] also described the preferrential accumulation of peg-functionalized micro particles in the inflamed gut mucosa of a human compared to the healthy human and other formulations, used in the study. However, these findings are somewhat contradictory to the size dependent deposition study which had been conducted so far in rats and where the

inflammation-induced accumulation was most pronounced for nano rather than micro particles [78]. Thus further species comparative studies appear necessary. The reason for this discrepancy between the micro and nano size remains hitherto unknown and requires further investigations. Effects observed with nanoparticles in rather small animals are obvious but are not easily translatable to humans, although the principle of targeting inflamed mucosal areas by particulate carriers is essentially valid.

These differences become essential when translating nanomedicine from animals to humans as the situation in human patients is much more complex than the animal models, therefore passive targeting alone may not be sufficient to optimize the therapeutic outcome. Active targeting strategies looking for luminal markers of epithelial inflammation are being discussed in literature e.g. utilizing apical expression of transferrin receptors in the inflamed intestine [125]. Alternatively, mucus targeting could be combined with size dependent accumulation e.g. in a multistage nanoparticle/microparticles-in-microsphere approach.

More in depth mechanistic studies using human patients and resected inflamed human intestinal tissue are needed to direct research efforts, a task which can only be undertaken in collaborations between pharmaceutical and clinical scientists. Targeted drug delivery to the site of action is one of the major attractions in nanomedicine. In the context of IBD therapy, the size-dependent accumulation of nanocarriers in the inflamed tissue can be utilized to increase therapeutic efficacy, reduce adverse effects and open up new delivery routes for fragile (bio) molecules. Still, better understanding of the mechanism behind the inflammation-induced accumulation phenomenon is needed to transfer this targeting concept successfully into the clinic.

1.4 Aim of the thesis and experimental design

As discussed in detail in the introduction part of the thesis, nanoparticles have been shown promising and encouraging approaches for the therapy of inflammatory bowel disease. The main aim of the thesis was to design a nanoparticle based carrier system which could deliver its cargo to the desired site, to minimize the adverse effects and to improve the therapeutic efficacy of the loaded drug. Thus, a biodegradable and biocompatible polymer poly(lactic-co-glycolic acid) (PLGA) was selected and nanoparticles were prepared by O/W emulsion evap-

oration method. Budesonide from the class of glucocorticoids was chosen as a model drug and was loaded into PLGA nanoparticles. Budesonide has a strong affinity to the corticosteroid receptors and the systemic bioavailability of budesonide is very low due to extensive metabolism in the liver, thus only 10% to 15% drug reaches to systemic circulation after oral administration. Therefore, a carrier system that could deliver budesonide directly to the target site was desirable. In addition, a pH-sensitive nanoparticle for colon-specific delivery was also required. An ideal carrier system for colon should retard drug release at acidic pH and allow the drug release at neutral or slightly alkaline pH. Thus, a pH-sensitive methylmethacrylate-copolymer (Eudragit® S100) was selected to protect budesonide loaded PLGA nanoparticles from early drug release in the stomach. After a physicochemical characterization, therapeutic efficacy of the designed carrier system on artificially induced colitis animal models was also essential. In order to achieve the aimed research objectives, the rest of the thesis was designed as follow:

- ◇ First of all budesonide loaded PLGA nanoparticles were prepared by O/W emulsion evaporation techniques and characterized for their size, shape, drug loading and drug release. The formulation was afterward tested on an *in vitro* 3D cell-culture model of the inflamed intestinal mucosa in comparison to the Liposome formulation, developed by Leonard et al., 2010.

- ◇ Secondly a novel Eudragit® S100 coated PLGA nanoparticles for colon targeted drug delivery was also prepared by emulsion evaporation techniques but with a slight modification and characterized. After *in vitro* characterization and stability tests, *in vivo* study was also evaluated in different acute and chronic colitis mice models. The results demonstrated better therapeutic activity for coated nanoparticles as compared to the free drug solution and uncoated nanoparticles.

Chapter 2

Methodological Approaches

2.1 Methods of nanoparticle preparation

A variety of material such as proteins [126], polysaccharides [127, 128], natural and synthetic polymers [129] can be used for the preparation of nanoparticles. The selection of matrix material depends on, required size, physicochemical properties of drug, surface charge, biodegradability, biocompatibility, toxicity, drug release from the matrix and antigenicity of the particles [130]. Mostly nanoparticles have been prepared by three methods: (1) dispersion of preformed polymer (2) polymerization of monomers (3) ionic gelation or co-acervation. However, other methods which has been described in the literature for the production of nanoparticles are supercritical fluid technology [131] and particle replication in non-wetting templates [132].

2.1.1 Dispersion of preformed polymer

This is a common method for the preparation of nanoparticles, mainly used to prepare nanoparticles from a biodegradable and biocompatible polymers such as poly (lactic acid) (PLA), poly (D,L-glycolide) (PLG), poly (D,L-lactide-co-glycolide) (PLGA) and poly (cyanoacrylate) (PCA) [72, 133–135]. This technique can be used by various ways but here only the most relevant methods are addressed.

Emulsion solvent evaporation method. This technique is based on oil in water emulsion and mainly suitable for encapsulation of lipophilic drugs [136]. In this method, the polymer is dissolved along with the drug in water immiscible organic solvent like dichloromethane, chloroform, methylene chloride or ethylacetate [137]. The mixture of polymer and drug is emulsified with an aqueous phase to form the oil in water (O/W) emulsion, containing a surfactant to stabilize the emulsion. The commonly used surfactants are polyvinyl alcohol (PVA) and polysorbates in concentration range from 0.1% to 10%. After the formation of emulsion the organic solvents can be evaporated or removed either by continuous stirring, reduced pressure or by increase of temperature. After complete evaporation of organic solvent the precipitation of polymer in water leads to nanoparticles. The water in oil in water (W/O/W) emulsion is the modified form of this technique and has been used for the encapsulation of water soluble drugs [138, 139]. In-order to produce small and monodisperse nanoparticles high speed homogenization or ultrasonication can be used [140].

Spontaneous emulsification or diffusion method. This is the modified version of the emulsion solvent evaporation method [141, 142]. In this method, the water miscible solvent like acetone or methanol along with the water immiscible organic solvent like chloroform or dichloromethane is used as an oil phase. Due to the spontaneous diffusion of water miscible solvent an interfacial turbulence is created between the two phases leading to the formation of small sized particles. As the concentration of water soluble solvent increase, a decrease in particle size can be achieved.

Salting out or emulsion diffusion method. The already discussed two methods require organic solvents which are hazardous to the environment and to the physiologic system as well [143]. Because of this safety and toxicity issues, Allemann and co-workers have developed a new method for the preparation of nanoparticles and named them salting-out method [144]. In this method two types of solutions, electrolyte saturated and nonelectrolyte saturated solution of PVA were used as a stabilizers and viscosity increasing agents.

Supercritical fluid technology. Production of nanoparticles by supercritical fluid technology now became an attractive alternative as it is an environmentally safe and cost effective for large scale production. The two most commonly used methods for the production of nanoparticles by supercritical fluid technology are rapid expansion of supercritical solution (RESS) and supercritical anti-solvent (SAS) method [134, 145].

Nanoprecipitation. The nanoprecipitation method in comparison with the other available method is more facile, less energy consuming and widely applicable technique for nanoparticle preparation. In this method, solvents should be miscible with the surfactant solution like acetone, ethanol or mixture of these two. The polymer along with lipophilic drug are dissolved in the water miscible solvent and added to the non-solvent under continuous stirring. The rapid diffusion of polymer solution in to the aqueous phase leads to the precipitation of nanoparticles [146].

2.1.2 Polymerization of monomers

In this method, nanoparticles are prepared by polymerization of monomers. The drug can be encapsulated either during the preparation of polymers or by adsorption after preparation of nanoparticles. Afterwards, the nanoparticles can be purified to remove the free drug residue or access of surfactant used during the preparation by centrifugation or by flow fil-

tration method. In another method [147] cyanoacrylate monomer and drug were dissolved in a mixture of an oil and absolute ethanol which was then added through a needle into an aqueous phase containing surfactant under continuous stirring and thus nanocapsules formed spontaneously by polymerization of cyanoacrylate. Nanoparticles prepared by polymeriazation techniques are, poly (ethylcyanoacrylate) (PECA) has been used for the encapsulation of insulin [148], poly (iosbutylcyanoacrylate) and oligonucleotide have been used for the encapsulation of Lipiodol [149, 150] and poly (isohexylcynoacrylate) has also been used for the encapsulation of phthalocyanine derivatives [151].

2.1.3 Co-acervation or ionic gelation

Research has been focused now on the preparation of nanoparticles using hydrophilic polymers like chitosan, sodium alginate, gelatin etc. in contrast to hydrophilic polymers. In this context, Calvo et al have developed a method for hydrophilic chitosan nanoparticles by ionic gelation [152, 153]. Briefly, the method involves a mixture of two aqueous phases of which one contains chitosan and a diblock copolymer of ethylene oxide (EO) and the other contains polyanion sodium tripolyphosphate (TPP). The basic mechanism of this method is that the positive charged amino groups of chitosan interact with the negative charged of tripolyphosphate to form coacervate. Due to electrostatic interaction between the two aqueous phases result coacervates, while ionic gelation occuring due to ionic interaction, involves the materials undergoing transition from liquid to gel at room temperature. The size of nanoparticles prepared by this technique ranges from 200-1000 nm with positive zeta potential.

2.2 Physico-chemical characterizations of nanoparticles

The nanoparticles can be characterized on the bases of size, surface charge and morphology, such physicochemical parameters are also important for the interaction of nanoparticles with the biological systems. Various techniques for the analysis of size and morphology are being in used like photon correlation spectroscopy (PCS), atomic force microscopy and electron microscopy. Another important parameter is the surface charge or zeta potential which is due to the electrical potential at the slipping plane of a particle moving in an electrolyte solution. The zeta potential shows charge on the surface of particles and moreover the exact value of

zeta potential can be used to predict the stability of nanoparticles dispersion [154].

2.3 Drug release from the loaded particles

To develop a successful and potential drug delivery system, drug release from the nanoparticles is one of the important parameter. Generally the rate of drug release from the matrix system depends on the solubility of drug, desorption of drug either surface bound or adsorbed drug, drug diffusion through particle matrix, erosion or degradation of nanoparticles matrix or a combination of diffusion and erosion of the matrix material govern release process [130].

The drug release from the nanospheres where the drug is uniformly distributed occurs either by diffusion or by erosion of the matrix, for example, if the drug release is faster than the erosion of the polymer used, then the release mechanisms are largely controlled by diffusion, or if the nanoparticle is coated by a polymer then the drug release from the core to release medium will also be controlled by diffusion process across the polymeric membrane. One common release profile of ordinary polymeric nanoparticles is the rapid or immediate release of drug. This rapid release in the first few hours of the release study which is also termed as 'burst' release is mainly due to the loosely attached or adsorbed drug on the surface of the particle [155]. The pattern of drug release mainly depends on the method of drug incorporation into a matrix system. For example if the drug has been loaded by incorporation method then the system will show relative low burst release and better sustained release. In case where the drug has been loaded into NP by adsorption method then the burst release will be high because the drug molecules are directly in-touch with the release medium therefore the drug could be immediately released. Fresta et al. [156] showed that drug loaded into NP by adsorption method, released the drug with high initial burst release up to 60% – 70% followed by a slow release, which demonstrates that the method of drug incorporation in to NP has a significant effect on the drug release.

Various methods have been reported for the in-vitro analysis of drug release profile which are: (1) side-by-side diffusion cells with artificial or biological membranes; (2) dialysis bag diffusion techniques; (3) reverse dialysis sac techniques; (4) ultracentrifugation; (5) ultrafiltration and (6) centrifugal ultrafiltration techniques. Though the standard method which has been reported in the United State Pharmacopeia (USP) is the dissolution test, however,

the paddle and basket have described as standard instruments. But the problem with these methods is the separation of nanoparticles from the release medium. Therefore two different methods exist, one method is to separate the nanoparticles from the suspension after sampling by centrifugation or by filtration. Second to separate nanoparticles release compartment from the sampling compartment by a dialysis membrane. This dialysis method has been considered to be more trustworthy then the centrifugation or filtration method because the released drug can equilibrate between the two compartments and the nanoparticles are too large then the pore size of the membrane and cannot cross the membrane [157].

2.4 Biopharmaceutical aspects

In drug delivery nanoparticles as a carrier system offers promising targeting application after administration. Nanoparticles can help in delivering of drug with poor solubility, low permeability or extensive first pass metabolism due to the specialized mechanism of up-take and absorption through the gastrointestinal tract. Various techniques have been reported to study nanoparticles up-take, bio-distribution and accumulation of nanoparticles at the target site, however few of them are discussed as follow.

2.4.1 Cell and tissue culture

Cell culture experiments can be used for the purpose of particles toxicity and their interaction with biological systems. Cell models have been provided an understanding about the effects of cytokines involved and other inflammatory mediators in the initiation and progression of the disease, apoptosis, injury and repair, necrosis and other process that may be involved in IBD [158].

Cell models have been considered an alternative model to the animal models but the drawback with such models is that it hardly reflects the complexity of the disease and is usually useful for single parameters. Therefore standard test are necessary to evaluate the NP toxicity such as MTT and lactate dehydrogenase (LDH) assays and also determination of cytokine response about cellular stress. The widely used cell lines in the research area of IBD are the Caco-2 cell line - a human colorectal cancer cell line - and the murine macrophages cell line RAW 264.7 [159]. Based on these cell lines, Tanoue et al. [160] developed an in-vitro model

and the anti-inflammatory effects of food factors were assessed. In their system, when the RAW264.7 cells were stimulated with lipopolysaccharide a decrease in transepithelial electrical resistance (TEER) value was observed and an increase in TNF-α production from RAW264.7 cells and an expression of IL-8 mRNA in Caco-2 cells. Their results showed that this model could mimic in-vivo the gut inflammation.

Another three-dimensional coculture model has been developed by Leonard et al. [119], basically the model has two main parts; apical compartments consist of Caco-2 cells while the basolateral compartment consists of blood derived macrophage and dendritic cells. In this 3-D cell model, macrophages and dendritic cells derived from peripheral blood monocytes were seeded into a collagen matrix and Caco-2 cells were seeded on top of collagen matrix and cultivated for 21 days. Afterwards, the inflammation was induced by different proinflammatory stimuli but only IL-1β was found to be a strong stimulator for Caco-2 cells. The system could show more complexity and information compared to the single cell model and could be a valuable tool for the testing and development of drugs and formulation for the therapy of IBD.

2.4.2 Nanoparticles cytotoxicity study

The research and use of nanoparticles are expanding day by day and the human exposure to nanoparticles is also inevitable therefore nanotoxicology research is now gaining attention. The properties of nanoparticles and their effect on human body are crucial before clinical use. For nanoparticles it is important to know that what are the factors which can influence the toxicity of nanoparticle so that their undesirable properties can be avoided, before moving to the clinical arena. Different tests have been evaluated and reported [161] for the toxicity of nanoparticle but the most relevant amongst them are Lactate dehydrogenase (LDH) and MTT 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide.

LDH is an enzyme which is normally released by damaged cells [162], thus its detection in cells supernatant is an indicator for damaged or lysed cell. LDH oxidizes lactate to pyruvate which then accelerates the conversion of tetrazolium salt INT to farmazan. The amount of LDH is directly proportional to the number of damaged cell [163]. In addition, to differentiate between the live and dead cells, the most widely used test is the MTT viability test [164]. MTT is a pale yellow dye which produces a dark-blue farmazan product only in viable cells

because the transformation is only possible in living cells. The amount of blue farmazan can be spectrophotometrically determined at 570 nm.

2.4.3 Nanoparticle and cell interaction

Interaction of nanoparticles with cells have been remained an important part of research in the field of nanoparticulate drug delivery. Since the last decade the interactions of nanoparticles with cell have extensively been studied. In the context of particles up take by phagocytic cells, the physicochemical characteristic for example size and surface of particles have been evaluated to play a vital role in their interaction with epithelial barriers [165–169]. Furthermore, size, shape and surface has been shown to be essential and the most influencing parameters regarding their interaction with cells, as the smaller size particles is often correlated with a greater extent of up take [170–172].

The main internalization pathways are phagocytosis, endocytosis or macropin-ocytosis, may occur according to the physicochemical properties of the nanocarrier and the nature of target cells. The endocytosis may be either clathrin mediated or caveolae mediated endocytosis [173]. The already mentioned pathways show different size specificities and also different localization of the internalized particles. It has also been reported that phagocytosis and macropinocytosis are slightly size specific and favor large size particles [165, 174], whereas clathrin mediated endocytosis has size specificity for particles less than 200 nm and caveolae mediated endocytosis has size specificity for less than 500 nm [175, 176]. In another study, Lai et al. reported that particles having size less than 40 nm are internalized by a nonclathrin and noncaveolae mediated pathway [177].

A study reported by Leonard et al [120], in their study the authors described in details the interaction of different types of nanoparticles with inflamed and healthy cells. In their developed 3D cell cultured model, inflammation was induced by cytokine IL-1β which were then quantified by the up regulation expression of interleukin-8 (IL-8) or TNF-α. The developed 3D model could provide more complexity and pathophysiological changes as compared to single cell model. The budesonide loaded PLGA and liposomes nano-formulations were afterwords tested for therapeutic efficiency in comparison to the free drug solution. The incubation time was 4 hours, approximate to the time in the intestine of IBD patients. Their results showed that budesonide loaded PLGA nanoparticles showed faster recovery of the TEER value and

superior therapeutic effects in controlling the IL-8 release in comparison to the free drug solution and budesonide loaded liposomes formulation. Further, their nanoparticles deposition study showed that budesonide loaded PLGA nanoparticles were observed in the junctional space of the monlayer and no co-localization with immunocompetent cells was observed.

Chapter 3

Budesonide Loaded Nanoparticles with pH-sensitive Coating for Improved Mucosal Targeting in Inflammatory Bowel Disease

Parts of this chapter are planned for a future publication as journal article.

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The author of the thesis designed the experiments, prepared and characterized the nano-formulations and wrote the manuscript.

3.1 Abstract

The purpose of this study was to investigate the therapeutic potential of budesonide loaded nanocarriers for the treatment of inflammatory bowel disease (IBD). First, budesonide was encapsulated in Poly (lactic-co-glycolic) acid (PLGA) nanoparticles by an oil in water (O/W) emulsion technique. A second batch of the same nanoparticles was additionally coated with a pH sensitive methyl-methacrylate-copolymer. The particle sizes of the plain and the coated PLGA were 200 nm and 240 nm, respectively. As could be shown *in vitro*, the pH sensitive coating prevented premature drug release at acidic pH and only releases the drug at neutral to slightly alkaline pH. The efficacy of both coated and plain nanoparticle formulations was assessed in different acute and chronic colitis mice models, also in comparison to the free drug. It was found that the coated PLGA nanoparticles alleviated the induced colitis significantly better than the plain particles, which were already more effective than treatment with the same dose of the free drug. These data further corroborate the potential of polymeric nanocarriers for targeted drug delivery to the inflamed intestinal mucosa, and that this concept can still be further improved regarding the oral route of administration by implementing pH–dependent drug release characteristics.

3.2 Introduction

Crohn's disease (CD) and ulcerative colitis (UC) are the two main types of idiopathic inflammatory bowel disease (IBD) [178]. The etiology of these disorders is not yet completely understood, however, several parameters such as environmental and genetic factors have been identified to contribute to the pathogenesis [179, 180]. The basic aim of IBD treatment is induction and maintenance of remission to achieve mucosal healing [181]. The currently available medications such as aminosalicylates, steroids and immunosuppressants are limited [27]. Moreover, the available carrier systems until date are also inappropriate for the selective IBD therapy [182]. An ideal system allows the delivery of adequate amounts of drug to the target site with adequate release kinetics.

Nanoparticulate based drug delivery have shown promising and encouraging approach in the therapy of IBD [5, 78]. Further, a study conducted on a rat in situ intestinal loop model, evidenced that nanoparticles (100 nm) are able to penetrate throughout the submucosal layers in comparison to the large size microparticles which mainly localized in the epithelial lying [183]. Also, the new endosomolytic bioreducible poly (amido amine disulfide) based nanoparticles have been proposed an efficient carrier for gene delivery [184–187] in the treatment of IBD. On the basis of these considerations nanoparticulate drug delivery system could be favorable for colon drug targeting in IBD.

However the oral delivery of these conventional micro or nano particles has some limitations such as initial burst release or lack of pH sensitivity which makes it less efficient for colon specific delivery. Additionally, biologic therapy such as anti TNF-α would greatly benefit from targeted delivery only to the inflamed mucosa. Also, the carrier systems reported until to date such as drug loaded nanoparticles, microparticles entrapped in enteric microparticles or matrix type pH sensitive system [89, 188, 189] are usually prepared by two steps of emulsification and solvent evaporation techniques which requires more efforts and time.

In this study, novel pH sensitive nanoparticles were prepared by single oil in water (O/W) emulsion evaporation techniques intended for colon targeted-drug delivery in IBD. The objective of this work was to minimize the early drug release in the gastric pH and to achieve a slow and sustained drug release in the distal part of the gastrointestinal tract (GIT) after oral administration which can be useful for colon-targeted delivery in IBD. The glucocorticoids

budesonide served as a model drug and was entrapped in PLGA core which was enterically coated with a methacrylate copolymer (Eudragit® S100). Budesonide has a strong affinity for glucocorticoid receptor [190] but due to extensive metabolism by cytochrome P-450 enzyme, very low amount drug reaches to systemic circulation [191]. Thus a carrier system for local delivery of budesonide was designed. As a comparison, budesonide loaded plain PLGA nanoparticles were also prepared by the same technique. After physical characterizations and evaluation of the novel carrier systems, the *in vivo* therapeutic efficacy was studied on TNBS, DSS and Oxazolone colitis mice models in order to strengthen the validity and effectiveness of the coated PLGA nanoparticles in comparison to free drug solution and as well as to plain PLGA nanoparticles.

3.3 Materials and methods

3.3.1 Preparation of plain and coated PLGA nanoparticles

Nanoparticles were prepared according to an emulsion solvents evaporation method [192]. The methodology in brief as follows: budesonide (0.2 % w/v) received from Sigma-Aldrich, Germany, was dissolved in 5 ml of organic solvent (ethyl acetate) containing 100 mg of poly (L-lactide-co-glycolide) (PLGA) (50:50 lactide : glycolide) provided by Evonik industries (Darmstadt, Germany). This solution of organic phase was thereafter added dropwise to an aqueous solution containing emulsifier (Poly (vinyl alcohol) (PVA, Mowiol® 4-88) MW 31000 was obtained from Sigma-Aldrich, Germany) through geared pump (Gilson Minipuls, France) under stirring. The emulsion was stirred for 2 hours at room temperature before homogenizing at 13,500 rpm for 10 minutes using an Ultra Turrax T-25 (Janke and Kunkle GmbH KG, Staufen, Germany). To this emulsion water was added and stirring was continued at room temperature to remove the organic solvents.

The same procedure with a slight modification was followed for coated PLGA nanoparticles. For enteric coating of PLGA nanoparticles, pH sensitive methyl methacrylate copolymer (Eudragit® S100) was purchased from Evonik industries (Darmstadt, Germany), (3 % w/v) was dissolved in 3 ml of 2 % PVA solution with an addition of 5ml methanol and 2ml of 0.1 N sodium hydroxide was also used to avoid precipitation of Eudragit® S100 in the external aqueous phase. The obtained polymeric drug solution was thereafter added drop wise to

aqueous phase under continuous stirring. The emulsion was stirred for 30 minutes at room temperature before sonication at 20 % amplitude for 2 minute using Probe Sonication (Branson Ultrasonic Shanghai, co., Ltd. PR china).

3.3.2 Physicochemical characterization

Particle size analysis

The particles size and size distribution were determined by photon correlation spectroscopy (PCS) using Malvern zetasizer 2000 HS (Malvern instrument, UK). Size measurement was performed in triplicate at room temperature and results were expressed in mean ± standard deviation (SD).

Scanning electron microscopy (SEM)

The external morphology of the nanoparticles was analyzed by scanning electron microscopy (SEM). A drop of nanoparticles was spread on silicon waver fixed with sample holder for SEM and coated after wards with gold using gold sputter in a high vacuum evaporator and observed on SEM JEOL 7000 F (Tokyo, Japan).

Determination of budesonide loading in plain and coated PLGA nanoparticles

The amount of budesonide entrapped within the nanoparticles (Table 3.1) was determined by measuring the free drug content in the supernatant after washing the nanoparticles three times with distilled water, using tangential flow apparatus. HPLC (UltiMate® 3000 from Dionex) method was used to determine the free drug content in supernatant. A reversed phase C18 Column (4.6 X 250 mm, pore size 5µm) (Merck KGaA, Germany) was used. The mobile phase consisted of a mixture of phosphate buffer (pH 3) and acetonitrile (60:40) delivered at a flow rate of 1.900 ml/min. Retention time was 6 min for each sample. The method was linear (r2 = 0.9997) over a range of 20 ng/ml to 500 µg/ml. The limit of detection was 0.01µg/ml and limit of quantification was 0.5µg/ml.

The drug content in PLGA and coated PLGA nanoparticles was determined by dissolving 5 mg of nanoparticles in 5 ml of ethyl acetate and acetone respectively. The ethyl acetate and acetone was then evaporated and after complete evaporation of these solvents the residue

was then dissolved in 5 ml of acetonitrile and phosphate buffer (4:6) pH3 for 2 hours. The same solution was then filtered through a disposable syringe filter paper (CHROMAFIL GF/PET 45/25) pore size 0.45 µm. 1ml from the clear filtrate was collected and were analyzed on HPLC as aforementioned.

In vitro drug release

In vitro drug release of the formulation was analyzed in simulated gastric fluid (SGF) of pH 1.2 and simulated intestinal fluid (SIF) of pH 7.4. These pH values were selected based upon the normal variation of gastrointestinal tract (GIT) in the stomach (pH 1.5), to the colon (pH 7 to 7.8) [193]. Simulated gastric fluid (SGF) of pH 1.2 (using 0.1 N HCl and 2gm NaCl for 1 liter) was prepared according to the United States Pharmacopeia (USP). Sample (5 mg) of the freeze dried PLGA or coated PLGA nanoparticles was weighted accurately and gently dispersed in 30 ml of SGF under stirring at a speed of 400 rpm and the release profile was continued for 2 hours. The pH of this medium was then changed with the addition of KH2PO4 (102 mg) and Na2HPO4.2H2O (155 mg) to the release profile medium to get colon sink condition, furthermore, 0.01M NaOH solution was used to adjust the pH to 7.4 and the release profile study was continued for 24 hours. 1.5 ml sample was withdrawn from the release profile medium at pre-determined time interval and centrifuged at 24400 g for 20 minutes. Subsequently, 1 ml sample from the supernatant was quantified by HPLC method as mentioned before.

Assessment of colonic inflammation

All animals used in the study were housed under specific pathogen-free conditions and experiments were performed in accordance with institutional guidelines. BALB/c mice between 8 and 12 weeks of age were obtained from the animal care facility at the University Clinic of Erlangen. All animal experiments were carried out in accordance with the German animal welfare act (TierSchG). Before induction of colitis all the mice were weighted and confirmed that they are healthy. For induction of colitis the most commonly used chemical agents such as TNBS, DSS or oxazolone was used with a modification of the previously described protocol citeWirtzC07. The animal were then grouped in to four sub-groups one group of mice were kept as untreated control, one was treated with free drug solution, one group were treated with plain PLGA nanoparticles and one were treated with coated PLGA nanoparticles. Each

treated group received orally an equal does of budesonide (dose: 0.168 mg/kg) either in the form free drug solution or nanoparticles suspension.

In vivo imaging of mice

For *in vivo* imaging of colitis activity, the imaging system IVIS 100 was used, which consists of a tight chamber equipped with a cooled CCD camera. The luminescent probe L-012 (Wako Chemical) was dissolved in sterile H2O to a final concentration of 20 mmol. L-012 was administered intraperitoneally in a 100 µl injection volume. During *in vivo* imaging the mice were immobilized upon administration of isoflurane (1.5%). Image exposure times were between 1 and 2 minutes, depending on the signal strength. Light emission from the region of interest was quantified as photons/second cm2/steradian. Tissues sample were obtained from all the treated and untreated groups and staining was done by immunohistochemical technique. First, cryosections from colon were PFA fixed and then staining of myeloperoxidase positive cells was done using a rat monoclonal antibody to MPO (Thermo Scientific) at a concentration of 1:100 and incubation for 10 hours at 4°C. Afterwards, slides were incubated with a Cy3 conjugated donkey-anti-rabbit secondary antibody (BioLegend), at a concentration of 1:200. Nuclei were stained with Hoechst 33342 (Life Technologies).

Histologic analysis of colon cross sections

Colon tissues specimen were obtained from colitis mice at indicated time points and embedded in cryosection which were then evaluated for mucosal architectural changes, inflammation, goblet cells depletion and signs of epithelial regeneration by haematoxylin and eosin staining followed by light microscopy (Olympus IX-70). The method in brief, 4 micrometer sections were made and stained with haematoxylin and eosin. For colitis induced by oxazolone, the degree of inflammation and epithelial injury on microscopic cross sections of the colon was graded semi quantitatively from 0 to 5 [194]. Grading of colitis activity was done in a blinded fashion by the same pathologist. Small bowel sections were taken from the same animals as an additional control and showed no evidence for inflammation.

Mini-endoscopic analysis

For monitoring of colitis activity, a high resolution video endoscopic system for mice was used [195]. The experimental endoscopy setup, denoted ''Coloview system'', consisted of a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple chip camera, and an air pump (Karl Storz, Tuttlingen, Germany) to achieve regulated inflation of the mouse colon. The endoscopic procedure was viewed on a color monitor and digitally recorded on tape. Prominent endoscopic signs of inflammation in mice were abrogation of the normal vascular pattern, the presence of mucosal granularity and the appearance of ulcers. To determine colitis activity the mice were monitored by miniendoscopy at indicated time points and murine endoscopic index of colitis severity (MEICS) [196] with five parameters (translucent, granularity, fibrin, vascularity and stool) was performed.

Determination of cytokine expression by qPCR

To better understand the severity and recovery of inflammation cytokines profile of a variety of markers was assessed in inflamed control and each treated groups. The treated mice received orally the same amount of budesonide either in the form free drug solution or as nanoparticles suspension. The quantitative real time PCR was used according to the manufacturer's guideline and the up and down in expression of cytokines was measured and is expressed as relative expression of 18 S-RNA (Fig 3.6).

Synthesis of cDNA from isolated mRNA by reverse transcription

Complementary DNA (cDNA) was synthesized after determination of RNA concentration using the SuperscriptTM III First-Strand Synthesis SuperMix for qt-PCR (Invitrogen). The manufacturer's guideline was followed for preparation of reverse transcription of RNA samples and was performed on ice. A volume equivalent to 1 µg of RNA as determined by UV spectrophotometric analysis was used for cDNA synthesis. Subsequently, quantitative real time PCR was used after dilution of cDNA samples to a final volume of 100 µl.

Quantitative analysis of cDNA by quantitative polymerase chain reaction

To determine the mRNA transcript level from the cDNA samples obtained from the large intestinal tissues, qualitative real time polymerase chain reaction (qPCR) was performed. The 18 s-RNA served as a control. Pre-diluted cDNA samples was mixed with 0.2 µM of primer pair detecting the murine TNF-α, 18 S-RNA, IL-6, IL-4 and IFN-γ and SYBR® Green PCR master mix followed by pipetting into an ABI prism 96-well optical reaction plate (Applied Biosystems). The qPCR reaction was performed in the C1000 Real-Time PCR System from Biorad using the following cycle program: 40 cycles 95 °C for 15 second and 60 °C for 1 min. comparative Ct analysis was used and results obtained from the PCR were analyzed to determine the relative amount of the murine TNF-α, 18 S-RNA, IL-6, IL-4 and IFN-γ.

Isolation of mRNA and real-time PCR

Total RNA was isolated from tumor and tumor free colonic tissue of mice with the RNA micro kit (Machery & Nagel) according to manufacturer´s guidelines. cDNA was subsequently generated with Affinity Script RT Multi-Temp RT (Agilent, Stratagene Products). Quantitative real-time PCR was performed with the QuantiTec SYBR Green PCR Kit (BioRad, peqlab) in combination with specific primers for IL-6, IFN-γ, IL-4 and TNF-α (Qiagen) on the iQ iCycler (Bio-Rad,Hercules,CA). Using beta-aktin or 18 S-RNA as a reference, the relative expression level of cytokine mRNA was calculated with the following formula: relative cytokine mRNA expression = 2$\wedge$[ct(cytokinecontrol) – ct(cytokinetreated) + ct(18 S-RNAtreated) – ct(18 S-RNA control), where ct is defined as the number of the cycle in which emission exceeds an arbitrarily defined threshold.

Localization of coated and plain PLGA nanoparticles

Instead of budesonide the hydrophobic fluorescent marker 6-cumarin was loaded into the coated PLGA nanoparticles to facilitate in-vivo detection after oral administration and prepared by same way as aforementioned. Whereas the plain PLGA nanoparticles were made up of fluorescence labeled PLGA polymer without budesonide. After induction of colitis both coated and plain PLGA nanoparticles suspension were administered by oral gavage (150 µl suspension). The mice were then euthanized after 18 h, the whole GI tract was isolated and

for detection of nanoparticle in the GI tract, spectral fluorescence images were obtained using the Maestro In Vivo Imaging System (CRI, Inc., Woburn, MA).

Statistical analysis

Statistical analysis of the in-vitro and in-vivo data was performed using SigmaPlot (SigmaPlot software, San Jose, California) and R software environment (R Development core Team 2012). Statistical difference between treatments (inflamed control and free budesonide, budesonide and budesonide loaded PLGA and between budesonide loaded PLGA and budesonide loaded coated PLGA nanoparticles groups) were determined by using A kruskal Wallis test and pair-wise Wilcoxon test for post-hoc. P values below 0.05* were considered as statistically significant.

3.4 Results

3.4.1 Preparation and characterization of nanoparticles

Preparation and characterization of nanoparticles Budesonide loaded plain PLGA nanoparticles were prepared by O/W emulsion techniques [192]. For additional protection from the acidic pH and enzymatic degradation during the gastrointestinal transit, pH sensitive coated nanoparticles were also prepared by the same techniques with a little modification. The particles size of the plain and coated PLGA nanoparticles were 200 nm and 240 nm, respectively (Table 3.1) with a narrow size distribution. Both, plain and coated PLGA nanoparticles showed high drug loading (85 – 90 %) with a production yield of 88 -91 %.

The morphology of the particles was investigated by scanning electron microscopy (SEM) (Fig 3.1). All particles displayed spherical shape and smooth surface. To analyze the coating of the nanoparticles, the particles were treated with two different organic solvents, ethyl acetate and methanol. Ethyl acetate is a solvent for PLGA but not for the coating, whereas methanol only dissolves the coating. SEM images of the particles recorded after solvent treatment in comparison to untreated particles (in water) revealed that the coated nanoparticles remained unchanged when treated with ethyl acetate due to the coating, while the plain nanoparticles collapse and lose their initial shape. Similarly, both particles were treated with methanol

and it was observed that the plain PLGA nanoparticles stayed unaffected while the coated nanoparticles aggregated, once the enteric coat was dissolved.

Formulation	Average particle diameter(nm)	Polydispersity index	Encapsulation efficiency % (indirect)	Encapsulation efficiency % (direct)
coated PLGA Nanoparticles	240 ±14.7	0.2 ±0.04	85 ±2.5%	88 ±0.30%
plain PLGA Nanoparticles	200 ±10.05	0.08 ±0.00	90 ±2.0%	90 ±0.20%

Table 3.1: Physicochemical characterization of budesonide loaded coated and plain PLGA nanoparticles

In vitro drug release testing was performed with both nanoparticle formulations in a pH progression medium for 24 hours; the initial pH 1.2 was changed to pH 7.4 after 2 hours (Fig 3.2). The plain nanoparticles showed an initial burst release followed by a slow release once reached to a plateau and no significant effect of pH change was observed as expected. In contrast, the coated nanoparticles exhibited a pH dependent release profile, showing a moderate release at acidic pH which ascended into a rapid release upon pH change to 7.4.

3.4.2 Colitis models

Three well established colitis animal models (DSS, TNBS and Oxazolone) [113, 197, 198] were selected for *in vivo* studies and the therapeutic efficiency of the free drug and drug loaded nanoparticles was tested. After induction of colitis, one group of animals were kept with inflamed mucosa for comparison while the other groups were then subjected to treatment either with free budesonide, budesonide loaded plain PLGA nanoparticles or coated PLGA nanoparticles.

3.4.3 Myeloperoxidase activity

Myeloperoxidase (MPO) is an endogenous enzyme in mammalian granulocytes and plays an important role in the initiation and progression of acute and chronic inflammation. In this study, the MPO activity was used as an index of granulocyte infiltration and was detected as a parameter for inflamed intestinal tissue in living mice as a versatile analytical alternative to

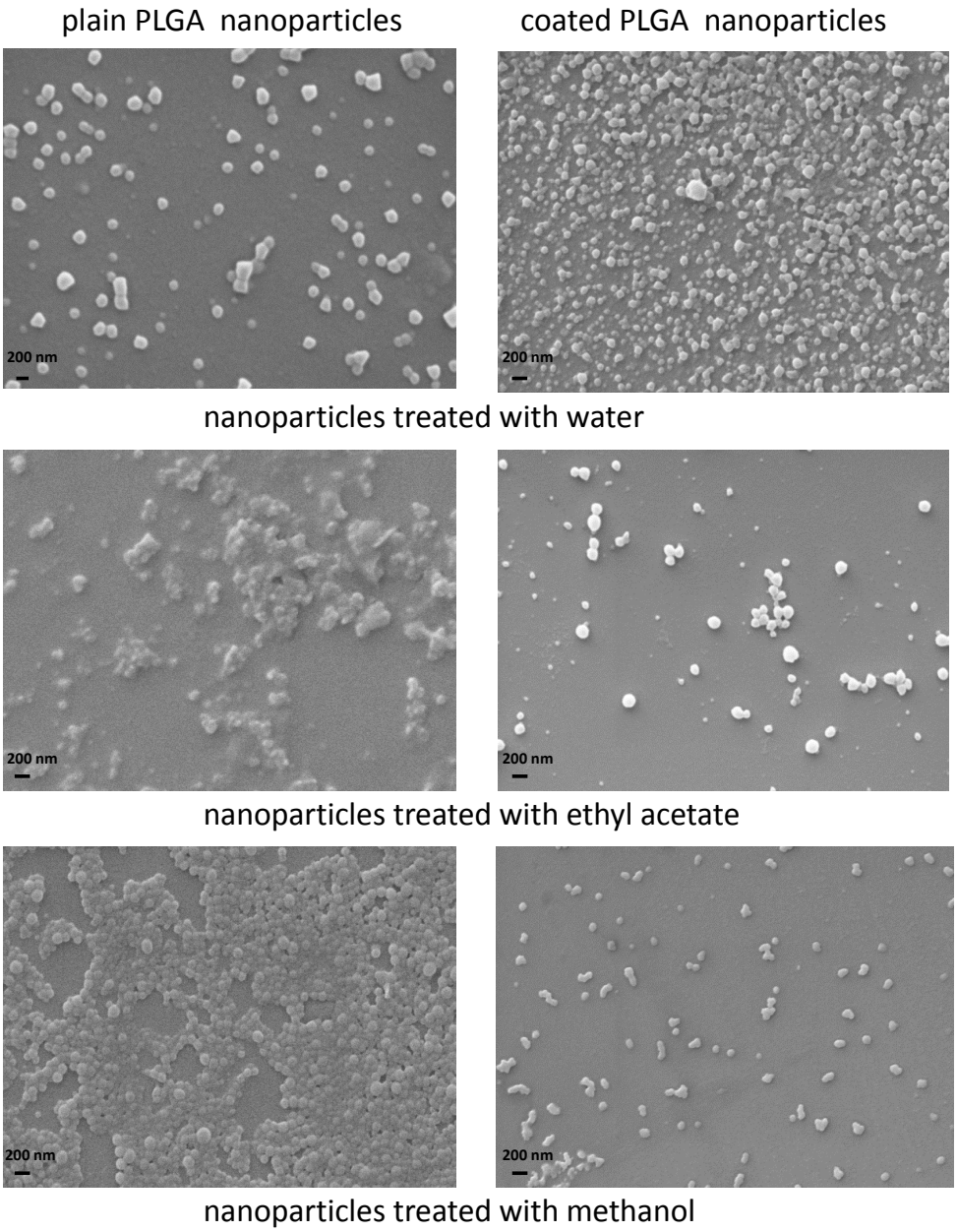


Figure 3.1: Scanning electron micrographs of budesonide loaded plain and coated PLGA nanoparticles.

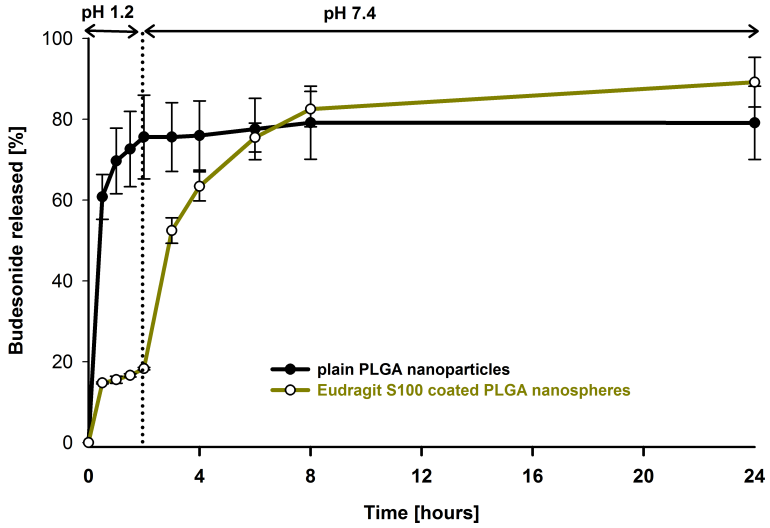


Figure 3.2: *In vitro* release of budesonide from plain and coated nanoparticles in simulated gastrointestinal fluid at pH 1.2 and 7.4. (mean, n = 3 ± SD)

existing methods as reported by Krawisz et al. and Xia et al. [199, 200]. Luminol (5- amino-2,3-dihydro-1,4-phthalazine-dione), a redox-sensitive compound, was used as a marker which upon exposure to an oxidizing agent emits blue luminescence (λmax=425 nm). After injecting luminal into the animals, they were placed in an *in vivo* imaging system 100 (IVIS) and bioluminescence signals were imaged by a charged coupled device (CCD) camera (Fig 3.3 (A)). Detection of the bioluminescent signal confirmed the induced colitis in the inflamed control group. In contrast, the mice either treated with free budesonide (Fig 3.3 (A) (b)) or budesonide loaded nanoparticles (Fig 3.3 (A) (c and d)) did not show any luminescence indicating that the inflammation has been recovered. Furthermore, these results were then confirmed by cryosectional study. Tissues samples were collected from all the groups for cyro-sectional study and stained by immunohistochemical techniques (Fig 3.3 (B)). The results obtained from the untreated group showed high extents of granulocytes and confirmed the severity of inflammation. However, as the study was in progress and when the treated groups were compared to the control, progressive decline in the number of granulocytes were observed. The group of animal treated with coated nanoparticles showed very low number of granulocytes and further confirmed the better therapeutic efficiency of the coated nanoparticles.

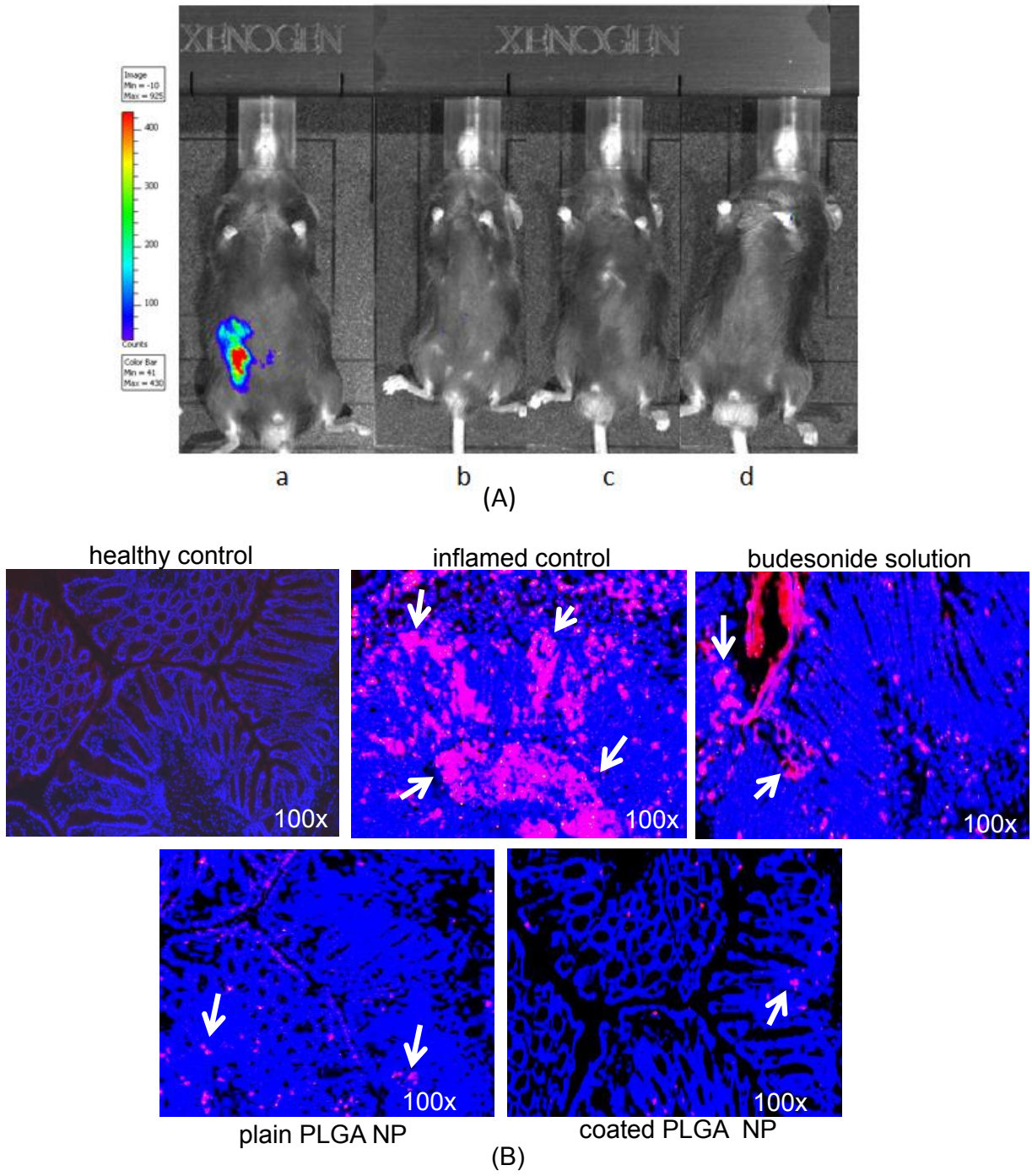


Figure 3.3: (A) *In vivo* MPO activity measurement in live mice by luminescence detection (a) inflamed colitis as control (b) free budesonide treated (c) treated with budesonide loaded plain nanoparticles (d) treated with budesonide loaded coated nanoparticles.(B) Cryosectional study by immunohistochemical techniques where the coated nanoparticles showed low extent of granulocytes.

3.4.4 Endoscopy and colitis score

A miniendoscopy study was performed on day 2 after induction of inflammation to check symptoms of the inflammation in live mice [195]. The results allowed monitoring and grading of disease as well as scoring of colitis. The inflamed mice clearly showed symptoms of inflammation with excessive mucus production and granular mucosa surface (Fig 3.4 (A)). Similarly, endoscopy study was also done for all the treated groups. Based on these endoscopic signs of inflammation a colitis score was developed ranging from 0 (no signs of inflammation) to 10 (severe colitis) (Fig 3.4 (B)). The results exhibited that coated PLGA nanopartilces worked better than the plain PLGA nanoparticles or free drug solution and showed lowest score in all colitis models.

All data were pooled for analysis by a Kruskal Wallis test for multiple comparisons and statistical significance difference (Fig 3.4 (C)). Overall, the results showed a progressive decline in colitis score when treated with free budesonide, budesonide loaded plain and coated PLGA nanoparticles. Furthermore, the data revealed that the group treated with free budesonide alleviated the inflammation (*$p<0.05$) when compared to the inflamed control. The group of animal treated with budesonide loaded plain PLGA nanoparticles demonstrated a decrease in inflammation (**$p<0.01$) compared to the free budesonide, whereas the group treated with coated PLGA nanoparticles showed lowest colitis score (***$p<0.001$) when compared to plain PLGA nanoparticles treated group and were of statistical significant difference. Coated PLGA nanoparticles exhibited comparatively better therapeutic activity due to their enteric coating allowing the delivery of a maximum amount of the loaded drug to the target site without prior lost in the stomach or other part of the gastrointestinal tract after oral administration.

3.4.5 Histological study and score

Changes in intestinal tissue histology are an important aspect to investigate feasibility and efficacy of the study upon induction of inflammation or treatment of inflammation. To evaluate such changes occurring at the histological level, haematoxylin-eosin (H&E) staining on tissue sections of the mice intestine was performed (Fig 3.5 (A)). All tissues specimens exhibited comparable histological features in all groups, however for the sake of space only one image of one animal per group is shown as a representative. The healthy control group showed nor-

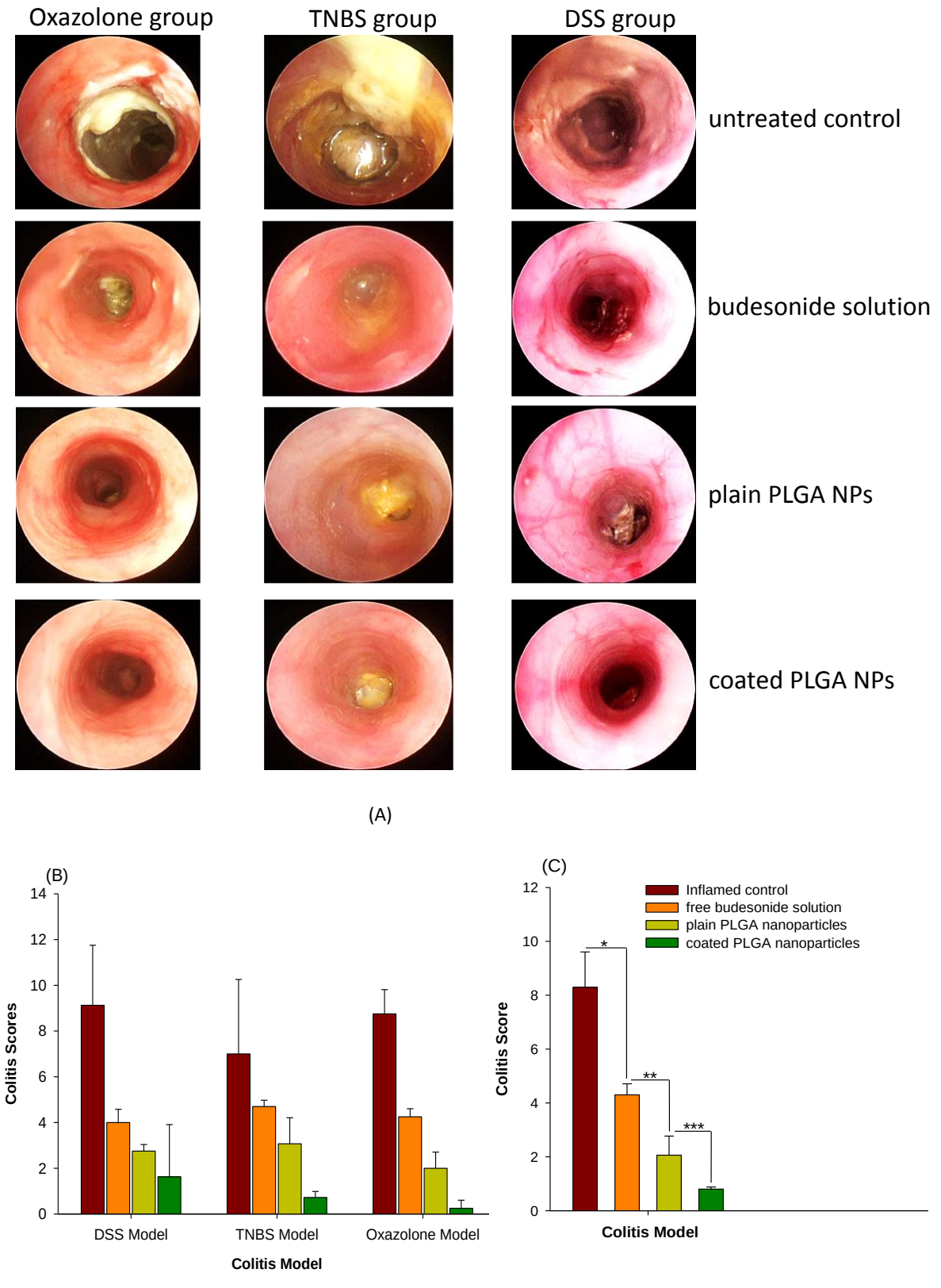


Figure 3.4: (A) high resolution miniendoscopy of healthy control, untreated inflamed and treated groups. (B) colitis score of all the three different animal models after treatment with free budesonide and budesonide loaded nanoparticles in comparison with the inflamed group (C) pooled data of all untreated and treated groups for statistical significance ($p < 0.01$ = **, $p < 0.001$ = ***).

mal colon histology without any signs of abnormal tissues architecture or disruption of tissues morphology. In contrast, tissues samples from the inflamed untreated groups exhibited clear signs of inflammation including goblet cells depletion, disruption and irregular mucosal structure. As the study progressed and animal treatment with formulations was continued the inflammation subsided substantially. Tissues section from the coated nanoparticles treated group demonstrated morphological tissue structure resembling that of healthy tissue indicating signs of epithelial cells restoration. These findings could be confirmed by histological scoring of the tissue sections as shown in (Fig 3.5 (B)). In the group treated with coated PLGA nanoparticles, the inflammation significantly alleviated (***$p < 0.001$) and the animals exhibited the lowest score compared to the other two treated groups which can potentially indicate the safe and effective delivery of the drug by coated nanoparticles.

3.4.6 Pro-inflammatory cytokines profile

As in IBD cytokines level are elevated, the expression profile of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and IFN-γ) in both treated and in inflamed control was measured by qPCR (Fig 3.6). All animals suffering from colitis in this study exhibited elevated levels of cytokines compared to the control group. Overall, the level of proinflammatory cytokines was substantially decreased in the treated groups when compared to the untreated colitis group. Further, the difference between the treated groups was not statistically significant. However, the group of animal treated with coated PLGA nanoparticles showed lowest concentration of proinflammatory cytokines in comparison to the other treated groups.

TNF-α has been considered a key mediator of inflammation and shares many proinflammatory activity therefore it was essential to evaluate the expression of TNF-α in colitis control and treated groups. The colitis control group showed highest level of TNF-α expression while the groups of animal treated with free budesonide, plain and coated PLGA nanoparticles exhibited downregulation in expression as shown in (Fig 3.6 (A)) The obtained results exhibited that the coated nanoparticles showed a statically significant difference ($p < 0.01$) when compared to the colitis control whereas the plain PLGA showed a statically significant difference ($p < 0.05$), while the difference between the free budesonide treated group and untreated group was statistically not significant.

The effect of interleukin-6 (IL-6) expression was also measured for all the groups and it was

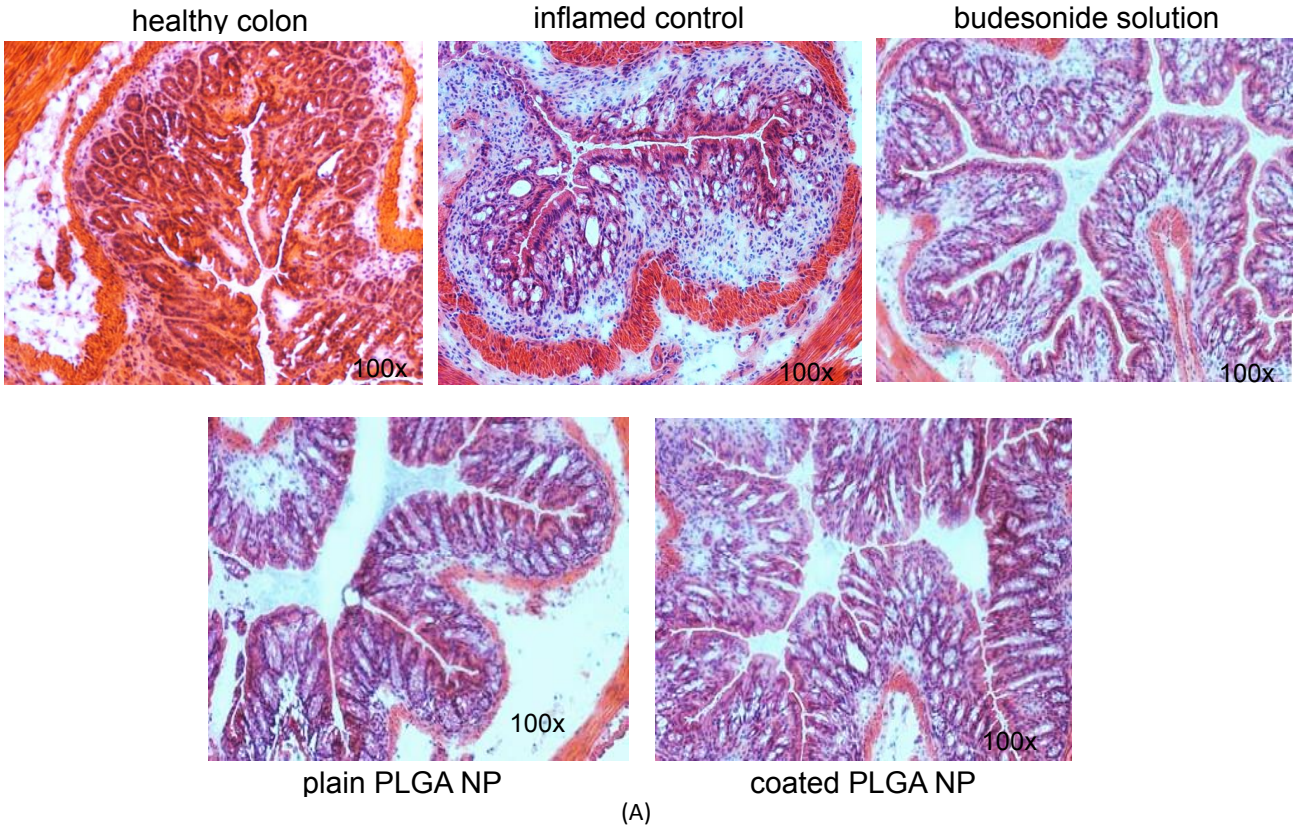


Figure 3.5: (A) Haematoxylin-eosin staining of the colon tissues isolated from healthy control, inflamed control and treated animals. Images of tissues cryosections obtained on day 2 of the experiment are shown with 100x magnification (B) Histological Score of the 4 groups. Each group consisted of 3 mice, only the coated PLGA nanoparticles showed a statistically significant difference ($p < 0.05$ = *, $p < 0.01$ = **, = $p < 0.001$ = ***) compared to the inflamed control.

observed that concentration of IL-6 was decreased gradually, when treated with free drug, plain PLGA and coated PLGA nanoparticles (Fig 3.6 (B)). However, the effect of coated PLGA nanoparticles on downregulation of IL-6 expression was statistically significant ($p < 0.001$) and exhibited the lowest IL-6 level amongst the treated groups. Similarly, it was also important to measure the expression level of IL-1β in both treated and inflamed groups. In this study it was observed that the level of IL-1β was up-regulated in inflamed control whereas in treated groups the expression of IL-1β considerably decreased (Fig 3.6 (C)). Further, we observed a statistically significant difference with a p value of 0.05, 0.01 and 0.001 for free budesonide drug solution, plain PLGA nanoparticles and coated PLGA nanoparticles, respectively.

The expression of interferon gamma (IFN-γ) was also evaluated in this study and observed a statistically significant difference. In contrast to colitis control the treated groups showed a 3.5, 4 and 6 fold downregulation in expression for free budesonide drug solution, plain PLGA nanoparticles and coated PLGA nanoparticles, respectively (Fig 3.6 (D)). Apart from the proinflammatory cytokines, interleukin-4 (IL-4) level was also measured. As it has been investigated that IL-4 is contra-inflammatory cytokines which limits monocytes and macrophages activation while there level is impaired in IBD citeRuckert96. In this study the colitis control group showed lowest level of IL-4 as was expected, while in the treated groups an increase in IL-4 level was observed. Though the difference between the treatment groups was not statistically significant for IL-4, however the coated PLGA nanoparticles showed highest level of expression (Fig 3.6 (E)).

3.4.7 Localization of coated and plain PLGA nanoparticles

To investigate the localization of coated and plain PLGA nanoparticles in the colon of healthy and inflamed groups, Maestro In Vivo Imaging System (CRI, Inc., Woburn, MA, USA) was used. Mice of all groups received an equal dose of nanoparticles by oral gavage. The study showed that in healthy mouse the nanoparticles are spread in the whole gastrointestinal (GI) tract while in case of inflamed mouse the particles are more abundant in the colon at the site of inflammation (Fig 3.7 (A)) as compared to the upper part of GI tract. This finding was further confirmed by cryo sectional study (Fig 3.7 (B)) for which confocal laser scanning microscopy (Carl Zeiss) was used. The results showed that the coated nanoparticles were found comparatively in higher quantities at the site of inflammation than the plain PLGA nanoparticles,

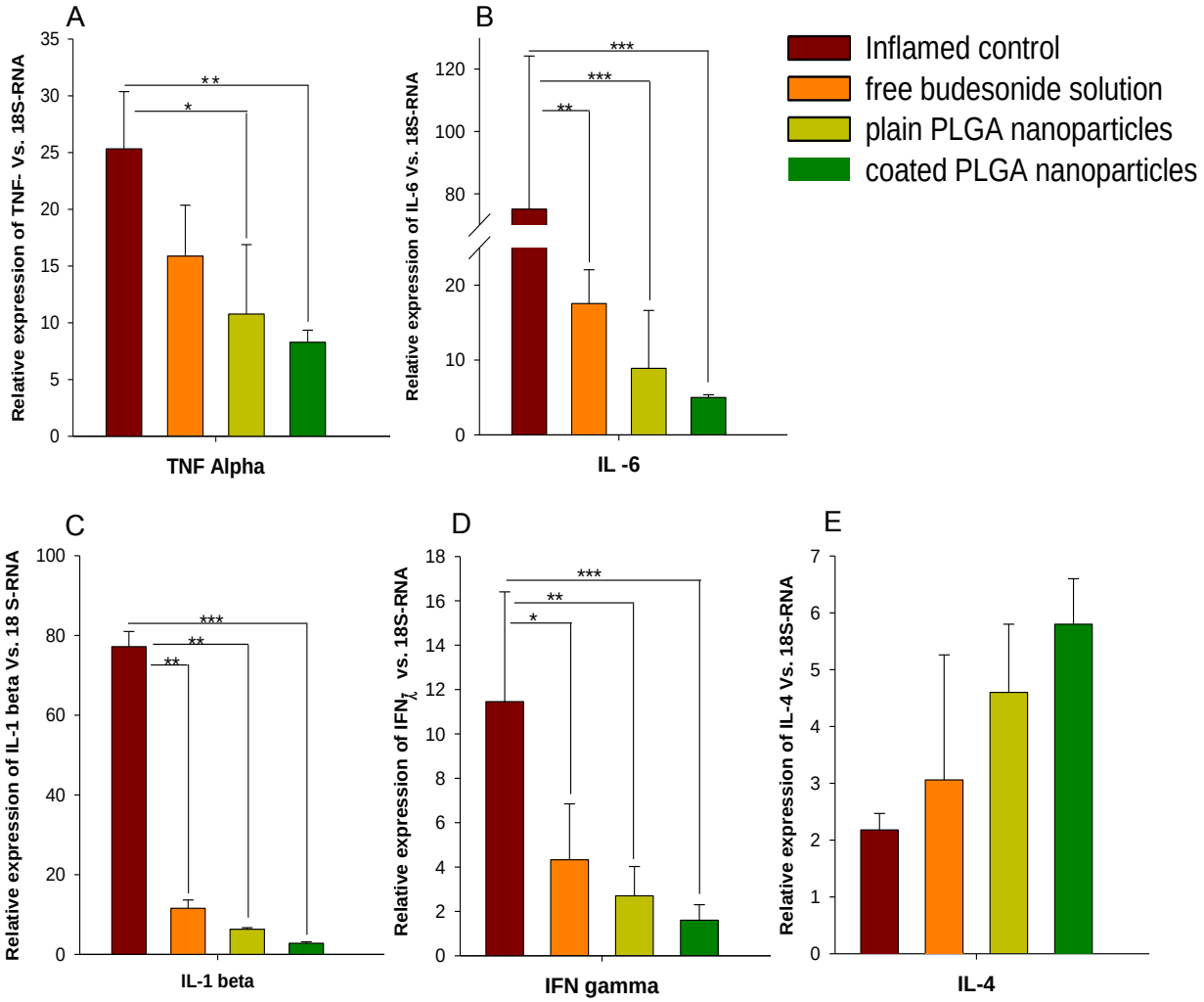


Figure 3.6: Average values of cytokines expression in inflamed colitis groups and treated groups. (A) tumor necrosis factor alpha (TNF-α) (B) interlukin-6 (IL-6) (C) Interleukin 1 beta (IL-β) (D) Interferon gamma (IFN-γ) (E) interlukin-4 (IL-4). Statistical comparison was performed on data set of inflamed control vs. free budesonide solution and coated and plain PLGA nanoparticle ($p < 0.05$ = *, $p < 0.01$ = **, $p < 0.001$ = ***)

which further support the hypothesis that small sized particles can accumulate to a greater extent at the site of inflammation.

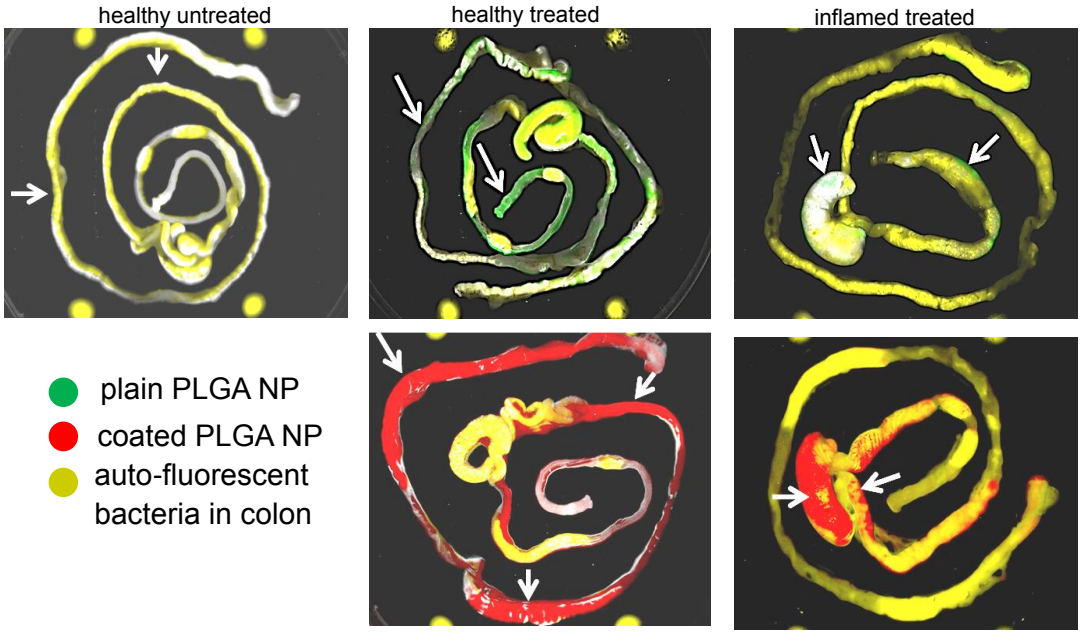
3.5 Blood Glucose Level

Since the locally acting glucocorticoid budesonide can enhance the intestinal sugar uptake and transport function of the intestine [201]. Therefore blood glucose level was also considered an important parameter for this study. As the obtained results in this studies showed that coated particles have the potency to delivery maximum amount of drug to the target site. Consequently a comparably high glucose level was expected from the coated particles to further confirm the specific target efficiency of the formulation at the site of inflammation (Fig 3.8). Mice of all groups were treated with an equal does of budesonide and Accu-chek performa test strips was used to measure the blood glucose level. The results showed a progressive raise in the glucose level when the treated groups were compared to the untreated control. However, the group of mice treated with coated nanoparticles showed significantly high level of glucose as compared to the plain nanoparticles and free drug solution treated groups.

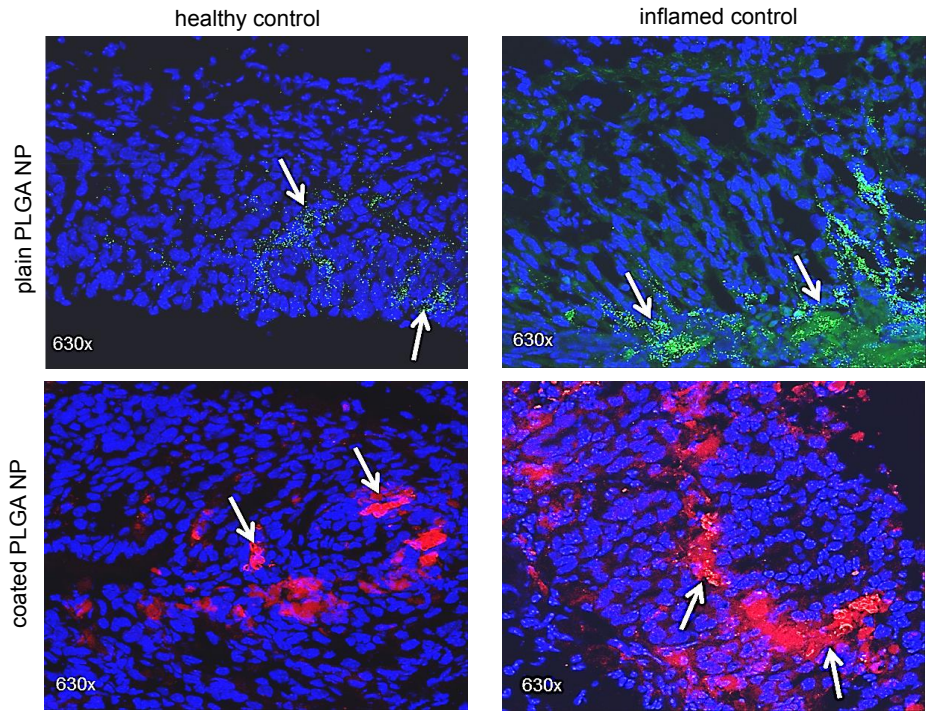
3.6 Discussion

In a previous study we showed that budesonide loaded plain PLGA nanoparticles exhibited improved therapeutic efficiency compared to other carriers, but due to lack of pH sensitivity such carrier system is less efficient for oral delivery [120]. Therefore an enteric coating was desirable to enhance the selective and maximum delivery of drug to the target site.

In this study, novel coated PLGA nanoparticles were designed for local IBD treatment, which combines the positive aspects of pH sensitive and sustained drug release. The basic aim of such combination was to minimize the early drug release in the upper part of the GIT by using an enteric polymer coating. Results obtained from the *in vitro* stability and drug release kinetics supported our hypothesis that, in contrast to the plain PLGA nanoparticles, the coated PLGA nanoparticles minimized the initial burst release at acidic pH and released the drug at neutral to slightly alkaline pH (Fig 3.2). However, the initial $\sim$18 % drug released at acidic



(A)



(B)

Figure 3.7: *(*A) In vivo localization of coated and plain nanoparticles in healthy control and inflamed control group. Negative control received no treatment. The red color shows 6-cumarin loaded coated nanoparticles while the green color is due to the fluorescence labeled plain PLGA nanoparticles. (B) cryosectional study showed the location of nanoparticles in healthy and inflamed mice.

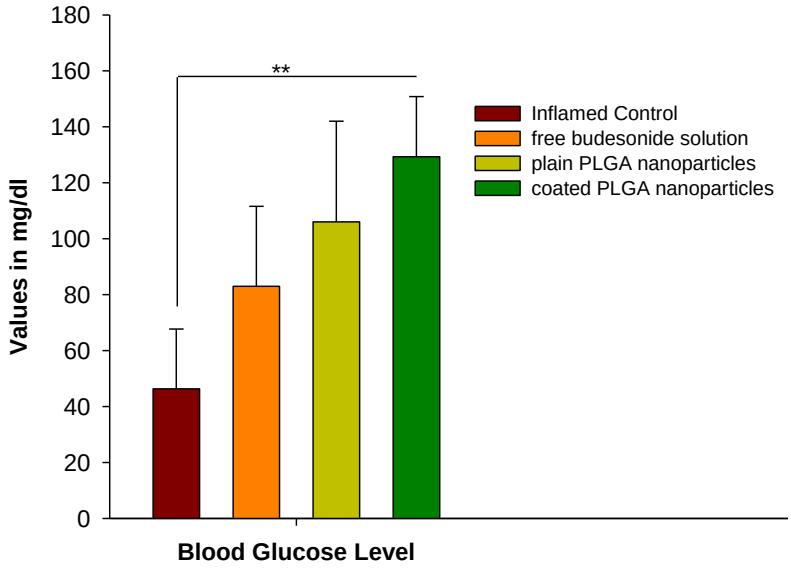


Figure 3.8: Blood glucose level of the animal treated with free budesonide solution, plain PLGA and coated PLGA nanoparticles ($p < 0.01$ = **).

pH from the coated PLGA nanoparticles might be due to the drug molecules which are not completely encapsulated in the coat shell but are on the surface of the particles. Besides, the coated formulation represents a stable and effective drug carrier system for colon delivery. Such carrier system which holds the initial burst release eventually minimizes adverse effects of the drug loaded and facilitates localize treatment via oral administration.

In all treated groups regardless of colitis model, treatment with coated PLGA nanoparticles significantly alleviated the induced colitis and moreover, general appearance of the coated PLGA nanoparticles treated group was much closer to the healthy mice group with no sever signs of inflammation as opposed to the remaining treated groups (Fig 3.4 (A)). From our *in vivo* study it was observed that the group of mice treated with free budesonide ameliorates the induced inflammation but did not completely recover and in some cases some signs of inflammation were observed. This might be due to extensive hepatic metabolism of budesonide before reaching to systemic circulation [202–205]. Mice treated with budesonide loaded PLGA and pH sensitive formulations significantly alleviated the induced colitis indicating superior therapeutic efficiency over free budesonide. Furthermore, when the coated PLGA nanoparticles were compared to the plain PLGA nanoparticles, in all cases it was observed that coated particles exhibited better therapeutic efficiency and the difference was

statistically significant.

For evaluation of therapeutic efficiency, the will established colitis models such as DSS, TNBS and oxazolone was used. The purpose was to test the novel nano-formulation on both, acute and chronic colitis animal models, because of pathophysiological differences such as clinical signs and immunological responses which exist in colitis models are differ from model to model [206]. Also it has been noted that TNBS induced colitis has similar histologic features to Crohn's disease while DSS induced colitis resembles to ulcerative colitis [207]. Therefore, such a comparative study was essential and interestingly it was observed from endoscopy, histologic study and cytokine profile that regardless of colitis model the formulation showed better therapeutic efficiency as compared to the free budesonide. Furthermore, the therapeutic efficiency of coated PLGA nanoparticles was comparatively better throughout in the study. The animal suffering from the induced colitis showed clear signs of inflammation and excessive mucus production as observed by mini-endoscopy (Fig 3.4 (A)) and irregular tissue architecture as observed by histological study (Fig 3.5 (A)). However, the treated groups resulted in alleviation of inflammation and showed signs of regeneration compared to the colitis-bearing groups.

On day 6 of the treatment, mice were euthanized and the colons were isolated for cytokines study as the activation of innate immune cells such as macrophages, monocytes and differentiated T cells in active inflamed area leads to the secretion of pro-inflammatory cytokines such as TNF-α. TNF-α exerts its proinflammatory effects through increased production of IL-1β and IL-6 [208]. Thus quantification of pro-inflammatory cytokines was essential and measured by qPCR. The results showed a significant downregulation of TNF-α, IL-6, IL-1β and IFN-γ expression. Both, the budesonide drug and budesonide loaded nanoparticles, alleviate the inflammation but the efficacy of coated PLGA nanoparticles was statistically significant in the study (Fig 3.6). Since budesonide has an effect on the suppression of TNF-α [209] but this better therapeutic effect could be attributed only to specific delivery of drug to site of action. The coated PLGA nanoparticles also significantly down-regulated the expression of IL-1β and showed better therapeutic efficacy as compared to other treated groups. Moreover, reduction in the expression of IFN-γ was also significant for coated PLGA nanoparticles in comparison to the other treated groups. In contrast to pro-inflammatory cytokines, anti-inflammatory cytokines such as IL-4 were also studied. IL-4 has immune-regulatory and anti-inflammatory

effect and plays important role in mucosal immunology [210] also level of IL-4 and IL-4 mRNA were found to be reduced in IBD [211]. Thus, after treatment up-regulation in the expression of IL-4 was observed as expected. Again, the group treated with coated PLGA nanoparticles showed better efficacy and comparatively high expression of IL-4.

Moreover, it has been investigated that nano-sized particles can preferentially accumulate in inflamed tissues and thus avoid rapid elimination by diarrhea [78]. In this study similar results were observed as reported in (Fig 3.7 (A and B)) and confirmed that the decrease in particle size increases the residence time. In healthy mice, it was observed that both plain and coated nanoparticles are dispersed in the whole GI tract while in inflamed mice the particles were found to a greater extent at the site of inflammation, highlighting the importance of particle size on colon targeting and indicating potential application of nanoparticles in the treatment of IBD. For further elucidation of local delivery, blood glucose level study was also evaluated to further confirm the site specific delivery of budesonide (Fig 3.8). The basic purpose of this study was to investigate the local action of budesonide on glucose up-take in mice with inflamed mucosa. A study reported by [201], described in detail that locally acting glucocorticoid enhances intestinal sugar up take. In their study the authors concluded that budesonide in comparison to prednisolone highly increased the blood glucose level. Similar to this study we also found a progressive increase in the blood glucose level after treatment with free budesonide; budesonide loaded plain PLGA and coated PLGA nanoparticles, respectively. Further, the animal treated with budesonide loaded coated nanoparticles showed highest blood glucose level as compared to the other two treated groups. This observation further supported the potential of coated nanoparticles and activity of entrapped drug over the free drug as well as over the plain PLGA nanoparticles.

3.7 Conclusion

The study exhibited that nanoparticles are better in the therapeutic efficiency than the free drug solution. Moreover, the nanoparticulate based drug delivery system can be further be improved by implement pH sensitive release characteristics as observed in this study. However further clinical studies and investigations are required to translate nanoparticulate carrier system and their associated advantages from laboratory into the clinic.

Chapter 4

Summary and Future Perspective

4.1 Summary and future perspective

It has been extensively demonstrated in published literature that nanoparticle-based drug delivery systems exhibit several advantages for the selective and safe treatment of IBD as compared to the available conventional therapy. In particular, the demonstrated accumulation of nanoparticles in inflamed mucosa shows great potential as the basis for targeted drug delivery for the therapy of IBD. Considering the importance of nanoparticles in the treatment of IBD, the aim of this study was to develop an oral nanoparticulate carrier system for the local delivery of budesonide for the treatment of IBD.

In this study, uncoated PLGA nanoparticles and Eudragit® S100 coated PLGA nanoparticles both loaded with budesonide were prepared by a modified emulsion evaporation technique. The particles were characterized and tested for stability before analysing their therapeutic efficacy both *in vitro* and *in vivo*. As a first step, the therapeutic efficacy of budesonide-loaded uncoated PLGA nanoparticles was tested in a 3D cell culture model mimicking the intestinal barrier in a state of inflammation. The obtained results showed that PLGA nanoparticles were superior in controlling the inflammation as compared to free budesonide and a budesonide-loaded liposome formulation, due to the formation of a drug depot. Further, the results demonstrated that application of the budesonide loaded PLGA nanoparticles resulted in a significantly greater down-regulation of expression of IL-8 compared to the free drug or budesonide-loaded liposomes.

In agreement with the obtained *in vitro* results, it could be shown that administration of both, uncoated and Eudragit® S100 coated PLGA nanoparticles to animals with experimentally-induced colitis led to a significant recovery of induced inflammation. Application of free budesonide was also shown to be effective, but to a lesser extent than budesonide delivered in uncoated or Eudragit® S100 coated PLGA nanoparticles. Comparison of the two nanoparticle formulations showed that the therapeutic efficacy of coated PLGA nanoparticles was superior to that of uncoated PLGA nanoparticles. This better therapeutic efficacy may be due to the enteric coat provided by the Eudragit® S100, which was shown in the current work to significantly retard the initial burst release of budesonide at acidic pH. Prevention of release of drug in an acid environment could be expected to result in delivery of a maximum amount of drug to the target site of the colon after oral administration.

In short, the studies conducted in this thesis describe the potency of nanoparticulate drug delivery systems for the treatment of IBD. Specifically, the studies illustrated that nanoparticles are an efficient carrier system for the safe and effective targeted delivery of budesonide for IBD treatment and can be used to achieve pH-dependent drug release characteristics. The obtained results in this study provide a background for basic understanding of nanoparticles and their significant role in the therapy of IBD.

As a future perspective, further investigations for clinically viable nano-carriers are essential. Also, based on these investigations, further clinical studies on human patients are required in order to achieve translation of nanoparticle-based therapeutics and their associated advantages from the laboratory into the clinic.

Chapter 5

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Scientific Contributions

Publications

Hussain Ali*, Benno Weigmann*, Eva-Maria Collnot, Maike Windbergs, Markus F. Neurath, Claus-Michael Lehr (2013), Budesonide loaded nanoparticles with pH-sensitive coating for improving the treatment of inflammatory bowel disease, *to be Submitted*.
(*shared first authorship)

Hussain Ali, Eva-Maria Collnot, Maike Windbergs, Claus-Michael Lehr (2012), Nano-medicines for the treatment of inflammatory bowel disease, *European Journal of Nano-medicine*. Volume 5, Issue 1, Pages 23–38, May 2013.

Eva-Maria Collnot, **Hussain Ali**, Claus-Michael Lehr, Nano- and microparticulate drug carriers for targeting of the inflamed intestinal mucosa, *Journal of Controlled Release*. Volume 161, Issue 2, Pages 235-246, July 2012.

Fransisca Leonard, **Hussain Ali**, Eva-Maria Collnot, Bart J. Crielaard, Twan Lammers, Gert Storm, Calus-Michael Lehr CM, In vitro screening of budesonide drug delivery systems for the treatment of IBD in a novel inflamed 3D cell culture model, *ALTEX*. 29(3):275-85, 2012

Conferences and Contributions

Poster presentations:

Hussain Ali, Christina Draheim, Claus-Michael Lehr, Eva-Maria Collnot, Novel drug delivery systems for the therapy of inflammatory bowel disease, 2nd International HIPS symposium, June 28, 2012, Saarland University, Germany.

Hussain Ali, Christina Draheim, Claus-Michael Lehr, Eva-Maria Collnot, Novel drug delivery systems for the therapy of inflammatory bowel disease, 9th International Conference on Biological Barriers, February – March 09, Saarbrücken, Germany.

Hussain Ali, Eva-Maria Collnot, Claus-Michael Lehr, In-vitro and in-vivo study of budesonide loaded PLGA NP with different release kinetics for the treatment of inflammatory bowel disease, CRS local chapter, March 15th 2011, Jena, Germany.

Hussain Ali, Martina Feistl, Eva-Maria Collnot, Claus-Michael Lehr Preparation and characterization of glucocorticoid loaded PLGA nanoparticles for the treatment of inflammatory bowel disease, 8th International conference on Biological Barriers, March 21 – April 01, 2010, Saarbrücken, Germany.

Workshop:

6th International GI-Targeting Workshop, May 9 - 10, 2012, Darmstadt, Germany.

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