Drug nanocrystals for the treatment of inflammatory bowel diseases (IBD)

Dissertation

zur

Erlangung des Doktorgrades (Dr. rer. nat.)

der

Mathematisch-Naturwissenschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

vorgelegt von

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aus

Kairo, Ägypten

Bonn, 2022

Angefertigt mit Genehmigung der Mathematisch-Naturwissenschaftlichen
Fakultät der Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der Promotion: 07.11.2022
Erscheinungsjahr: 2022

Die vorliegende Arbeit entstand unter der Leitung von

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Rheinischen Friedrich-Wilhelms-Universität Bonn

Printed and/or published with the support of the German Academic Exchange Service (DAAD)

Acknowledgement

To **Allah**, the Most Gracious and Merciful, for allowing me to do this work and giving me the knowledge, strength, and guidance to do something that can help people, as He says in the Holy Quran that the one who saves a life and lets it live on is as if he has saved all mankind. For all this and for his guidance through my life, I am very grateful.

I would like to express my heartfelt gratitude to my mentor and supervisor, ***Professor Alf Lamprecht***, for allowing me to conduct my PhD research in his laboratory. For his great and respectful way of advising and guiding me, his supportive supervision, and his willingness to let me participate in scientific conferences and publications, no words are sufficient to express my gratitude to him.

I am very thankful to ***Dr. Maryam A Shetab Boushehri***, for her professional help during this work, she always provided me with information, advice and suggestions even by telecommunication during the hard times of the pandemic. For her helpful and supportive attitude, I am very grateful.

I am very grateful to ***Dr. Mohamed Ehab Ali*** and ***Dr. John Mitri*** for their professional and personal support about this work and my life in Germany.

In addition, I would like to thank ***Dr. Brice Moulari*** for his efforts in the in vivo experiments, ***Thilo Faber*** for the electron microscopy imaging and the kind cooperation during the work on this dissertation, ***Martina Gerlitz*** for taking care of administrative matters, and ***Alexander Ramich*** for his great technical assistance.

I am also very thankful to all my colleagues in the Department of Pharmaceutics for their continuous help and encouragement especially to my dear officemates ***Dr. Jan Kozak Tugrul Mert Serim*** and ***Sahel Vahdati*** for the nice time we spent together, their help and advice to me during this work.

I would like to thank the Egyptian Ministry of Higher Education, the German Academic Exchange Service (DAAD) (91613363), the German Federal Ministry of Education and Research and the Ministry of Culture and Science of the State of North Rhine-Westphalia their financial support to my stay in Germany during this work.

I present my sincere gratitude, thanks and love to my ***Mother and Father*** who was and still always encouraging to complete my scientific research. Really. I can't express with words what they have done for me. May Allah bless them and have mercy upon them as they brought me up when I was small.

I would like to express my thanks, love, and appreciation to my beloved wife ***Yomnah Elsayed*** and my little kids ***Sara and Yaya*** for their continuous help and support during this work and for their patience on my continuous absence from home doing this thesis at lab.

Lastly, I would like to thank my two brothers ***Islam and Amr*** for their fast help and support in all issues in my life.

Abstract

Inflammatory bowel disease (IBD) is a group of chronic inflammatory diseases of the gastrointestinal tract that mainly includes Crohn's disease (CD) and ulcerative colitis (UC). Both diseases manifest similar symptoms such as persistent diarrhea, rectal bleeding, and abdominal pain. Drug treatment for IBD was and still is aimed at reducing the inflammatory state and treating the symptoms. Mesalazine/5-ASA was used for decades for mild or moderate colitis, and corticosteroids or immunosuppressants were used in severe cases. However, the progressive understanding of the pathophysiological mechanisms of IBD led to the development of more specific drugs that can break the vicious cycle of inflammation by neutralizing one of the inflammatory mediators, reducing the adhesion of leukocytes to some receptors overexpressed in the inflamed tissue, or inhibiting the transcription factors that lead to the synthesis of the inflammatory cytokines. A new category of drugs has been developed that target either the remodeling of the microenvironment of inflammation by inhibiting indirect proinflammatory factors (e.g., low-molecular-weight heparin and anticoagulants) or by stimulating the release of anti-inflammatory enzymes as a byproduct of their metabolism in the tissue (e.g., hemin). In theory, the more specific a drug is, the fewer adverse effects it has, but the complexity of the human body precludes this simple theoretical assumption. This is because drug targets that play a role in the inflammatory state play a very different role in normal state. In other words, the search for a new drug to treat IBD should be accompanied by extensive research into systems that can safely deliver these new drugs to their targets while sparing their off-target distribution.

Nanotechnology-based drug delivery depends on the use of a non-living biodegradable carrier that can be formulated as small particles and therefore passively accumulates in inflamed tissues. This carrier has specific physicochemical properties that allow it to incorporate the drug into its intraparticle structure. The size, surface charge, and shape of these nanoparticles (NPs) can be fine-tuned to achieve better accumulation in inflamed tissue than in non-inflamed tissue. However, the main challenge in optimal drug delivery through nanoparticles is to deliver the drug directly to the site of inflammation in sufficient quantity to exert its pharmacological effect. Although modification of the above factors could affect targeting and accumulation in the inflamed tissue, the maximum possible drug loading of NPs should be achieved.

Drug nanocrystals (NCs) consist mainly of nanoscale drug particles coated with a polymer and/or a surfactant and contain the highest amount of drug per particle. They have been studied for the treatment of inflammation, where they have shown great potential. However,

few studies have been conducted in the field of colitis treatment. In addition, the effects of particle size, surface charge, and particle shape of NPs on their efficacy in IBD have been studied in detail. However, few studies have been conducted to evaluate these effects on the efficacy of NCs.

The interdependence of inflammation and coagulation has enabled the development of new therapeutic strategies for IBD based on inhibition of the coagulation pathway. For example, rivaroxaban (RX), a factor Xa inhibitor, was able to reduce macroscopic and histological signs of inflammation in TNBS-induced colitis. It also showed antioxidant activity and decreased the accumulation of transforming growth factor β1 (TGF-β1) and malonyldialdehyde (MDA), as well as the activities of MPO, tissue inhibitor of metalloproteinases-1, and matrix metalloproteinase-3. However, nonspecific administration of RX to inflamed and noninflamed colonic tissues could lead to an unintended anticoagulant effect in the colon, which could exacerbate preexisting colonic bleeding associated with IBD. Therefore, selective administration of RX to inflamed tissue sites would be of great benefit to break the vicious cycle of inflammation and clotting at these sites and spare non-inflamed sites from the anticoagulant effect.

Therefore, fluorescent nano- and microcrystals (MCs), RX nano- and microcrystals, and RX nanocrystals with different surface charges were prepared by wet milling to evaluate their selectivity for inflamed tissue. To evaluate the formulation factors, i.e., HPMC and SDS concentration, drug to beads amount ratio, volume of stabilizer solution, milling bead diameter, and milling time, these factors were varied to produce fluorescent NCs with particle size in the range of 200-500 nm. This analysis showed that the most important factors were the concentration of the stabilizer solution, the diameter of the beads, and the milling time. Increasing the SDS concentration, decreasing the HPMC concentration, increasing the grinding time, and using larger grinding beads resulted in smaller NCs. However, the magnitude and effects of these factors vary from one study to another. Therefore, there are no optimal values that can be used in all cases, but they should be evaluated specifically for each study. The RX NCs were then prepared according to the optimal values for each factor. SEM images of the RX NCs showed that they had an irregular shape and a size between 200 and 500 nm. Differently charged NCs were formulated by using different surfactants and different milling time to achieve the smallest particle size, and anionic RX microcrystals were prepared by reducing the milling time and ball diameter.

In vitro evaluation of the anti-inflammatory activity of rivaroxaban NCs, MCs, and solution was performed on J774-DUAL™ macrophage cells after determining the safe concentrations that could be used by each formula by the MTT assay. It was found that the anionic nano- and microcrystals were the only formulations that caused a reduction in percent inhibition of secreted embryonic alkaline phosphatase (SEAP) amount in the culture media in J774-DUAL™ macrophage cells with a comparable value of approximately 54%. Other formulations resulted in a non-significant change in SEAP in inhibition.

The difference in bioadhesion between NCs and MCs to inflamed colon tissue was then determined by applying the fluorescent formulations rectally for three days to the colon of mice with TNBS-induced colitis. After sacrificing the animals, desiccation, and washing of the colon, fluorescence intensity was determined in inflamed and non-inflamed tissues. It was found that the fluorescence intensity in inflamed tissue was 13- and 15-fold higher than in noninflamed tissue for MCs and NCs, respectively.

To determine whether these bioadhesion and in vitro effects could be translated into an anti-inflammatory effect in vivo, the anionic NCs and RX microcrystals were tested in the TNBs colitis model, where they showed comparable anti-inflammatory effects in terms of their ability to reduce the clinical activity score (CAS) and weight/length ratio, as well as MPO and IL-1β levels in inflamed tissue. However, only anionic NCs at a dose of 5 mg/kg produced a significant reduction in TNF-α levels. Although the differences in MPO, TNF-α, and IL-1β levels between the groups at the same dose were not significant, the different level of their significance compared with the colitis model suggests a slight superiority of NCs over microcrystals in reducing inflammation.

Application of RX NCs with different surface charges in the TNBS colitis model revealed that anionic NCs had a stronger anti-inflammatory effect than nonionic and cationic NCs, as evidenced by their ability to decrease MPO (79.1± 11.9 U/g tissue) and TNF-α levels (497.1± 158.3 U/g tissue) in colonic tissue compared with the colitis control group (608.2± 241.5 and 1583± 492.1 U/g tissue, respectively). Furthermore, the assessment of factor Xa levels in blood showed that RX anionic nanocrystals produced levels lower than the levels produced with RX microcrystals, and solution, suggesting a systemic anticoagulation effect.

Due to the irregular shape of RX NCs, which resembles some polymeric NPs, it was not possible to determine whether NCs with a different shape from polymeric NPs could passively deliver the drug to the target or enhance the anti-inflammatory effect of the drug compared with its solution. Therefore, hemin was selected for the second study because hemin NCs are needle

shaped. Hemin solution has already been studied as a potential alternative to conventional IBD therapy and showed promising results. However, targeted use of hemin in inflamed tissues would be of great benefit for the treatment of IBD, as the effect could be enhanced, and the dose could be reduced.

Like rivaroxaban NCs, the hemin NCs were prepared depending on the optimal values of the preparation factors, which were determined previously. The particle size of the hemin NCs was approximately 150 nm, and SEM imaging confirmed the needle-like appearance of these NCs. They were then tested for their toxicity and anti-inflammatory effects on J774-DUAL™ macrophage cells, and the NCs showed lower toxicity and higher anti-inflammatory effects than the solution. In addition, at low concentrations, the hemin NCs showed no pro-inflammatory effect like the hemin solution.

In the TNBS colitis model, all the hemin formulations administered rectally caused a significant decrease in MPO levels compared with the colitis control group, indicating the superiority of rectal administration over intraperitoneal administration because of the local effect. In addition, hemin NCs at a dose of 10 mg/Kg had the best anti-inflammatory effect, as evidenced by their ability to reduce MPO levels (162.8± 65.6 U/g tissue) and TNF-α levels (606. 4± 320.5 U/g tissue) and IL-1β levels (657.7± 109.1 U/g tissue) compared with the colitis control group (608.2± 241.5, 1583± 492.1, and 1790± 386.3 U/g tissue, respectively). Moreover, hemin NCs at a dose of 5 mg/kg were able to achieve a comparable effect to the higher-dose hemin solution (10 mg/kg). Therefore, this study suggests that hemin can be used at a lower dose for the treatment of IBD when formulated as NCs than in solution. The presence of hemin NCs as needles suggests that the needle form of these NCs did not hinder but might rather enhanced the effect of hemin in the treatment of IBD.

From the previously described studies, it was concluded that formulation of drugs as NCs enhances their anti-inflammatory effect for IBD management. Their ease of preparation and scale-up and the ability to deliver higher amount of drug compared to polymeric or lipid-based NPs exploit the great potential of using drug nanocrystal technology in IBD, which has not been seriously considered to date. Furthermore, it was concluded that anionic NCs are the most effective type of NCs compared to non-ionic and cationic counterparts. The irregular forms of the prepared NCs have not impeded them from enhancing the effect of drug. Further studies are required to determine the effect of oral NCs or their gastroprotective coated equivalents in improving drug efficacy for the treatment of IBD.

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List of abbreviations

5-ASA	5- Aminosalycilic acid
ANOVA	Analysis of variance
CAS	Clinical activity score
CD	Crohn's disease
CLSM	Confocal Laser Scanning Microscope
CO	Carbon monoxide
CO_2	Carbon dioxide
CTAB	Cetyltrimethylammonium bromide
DAMPs	Damage/danger-associated molecular patterns
DCs	Dendritic cells
DLS	Dynamic light scattering
DMEM	Dulbecco's minimum essential medium
DMSO	Dimethylsulfoxide
DSS	Dextran sulfate sodium
ECM	Extracellular matrix
ELISA	Enzyme-linked Immunosorbent Assay
EPR	Enhanced permeation and retention
FBS	Fetal bovine serum
FITIC	Fluorescein isothiocyanate
FXa	Factor Xa
GIT	Gastrointestinal tract
HO-1	Heme Oxygenase-1
HPMC	Hydroxypropylmethylcellulose
IBD	Inflammatory bowel diseases
IBDU	Inflammatory bowel disease, type unclassified
IFN-ɣ	Interferon- ɣ
IL	Interleukin
IP	Intraperitoneal
LC_{50}	Lethal concentration 50
LDE	Laser doppler electrophoresis
LMWH	Low molecular weight Heparin
LPS	Lipopolysaccharide
LT	Leukotrienes

MCs	Microcrystals
MDA	Malonyldialdehyde
MPO	Myeloperoxidase
MPP	mucus-penetrating particles
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide
NCs	Nanocrystals
NF-κB	Nuclear factor kappa B
NPs	Nanoparticles
P188	Poloxamer 188
PAA	polyacrylic acid
PAM	polyallylamine
PAMPs	Pathogen-associated molecular patterns
PAR-2	Proteinase activated receptor-2
PDI	polydispersity index
PEG	Polyethylene glycol
PG	Prostaglandins
PLGA	Poly(lactic-co-glycolic acid)
PMA	Phorbol-12-myristate-13-acetate
PVP	Polyvinyl pyrrolidone
RES	The reticuloendothelial system
ROS	Reactive Oxygen species
RX	Rivaroxaban
SDS	Sodium lauryl sulfate
SEAP	Secreted embryonic alkaline phosphatase
SEM	Scanning electron microscopy
TGF-β	Transforming growth factor β
T_h	T-helper cells
TLRs	Toll-like receptors
TMC	Trimethyl chitosan
TNBS	Trinitrobenzenesulfonic acid
TNF-α	Tumor necrosis factor- α
T_{reg}	T-regulatory cells
UC	Ulcerative colitis

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1 Introduction (theoretical background)

1.1 Inflammatory bowel diseases (IBDs)

Published as a part of ***"Drug delivery to the inflamed intestinal mucosa – targeting technologies and human cell culture models for better therapies of IBD"*** in ***Advanced Drug Delivery Reviews***.

Olga Hartwig, Maryam A. Shetab Boushehri, Karim S. Shalaby, Brigitta Loretz, Alf Lamprecht, Claus-Michael Lehr, 2021.
Drug delivery to the inflamed intestinal mucosa - targeting technologies and human cell culture models for better therapies of IBD. Adv. Drug Deliv. Rev. 113828 (2021) doi:10.1016/j.addr.2021.113828.[1]

Inflammatory bowel diseases (IBDs) are a group of chronic, immune-mediated disorders of the gastrointestinal (GI) tract, comprising Crohn's disease (CD) and ulcerative colitis (UC) as main entities. The main diagnostic strategies for the disorder include the endo/colonoscopic and biopsy-mediated detection of the inflammation, complemented by computed tomography/ magnetic resonance imaging for the detection of the non-colonic inflammation sites. Both disorders share similar symptoms such as episodic flares with persistent diarrhea, rectal bleeding, and abdominal pain. Nonetheless, despite many shared clinical and pathological features associated with the exaggerated innate and acquired immune response as well as compromised epithelial barrier integrity, IBD is known as a heterogeneous disease, with marked differences in clinical presentation, genetic background, and response to treatment. In particular, the location of the inflammation is a distinguishing feature. UC is often restricted to the colon and is associated with continuous but more superficially inflamed tissue (Ordás, Eckmann, Talamini, Baumgart, & Sandborn, 2012). CD, on the other hand, may affect all parts of the GI tract in patchy distribution, can affect various gut layers, and may involve granulomas and crypt abscesses (Baumgart & Sandborn, 2012). Regardless of the type of the disorder, IBD results in significant morbidity and reduced quality of life for many patients. The onset of the disorder is often in adolescents or young adults, and given the lack of absolute cure, the best treatment options are still limited to long-term (preferably steroid-free) symptom alleviation and lifestyle modification (Magro et al., 2017). While the hitherto achieved improvements in medication and surgical care has shifted the life expectancy of the IBD patients closer to that of normal people (Ellen Kuenzig, Manuel, Donelle, & Benchimol, 2020), a substantial

[1] A summary of the publication with an indication of the doctoral student's contribution can be found at the end of this dissertation (section 8)

percentage of patients remain non-responsive to first- and second-line treatments (Peyrin-Biroulet & Lémann, 2011; Schreiner et al., 2019). Moreover, serious complications such as fissures, fistulas and strictures often necessitate surgical inventions (e.g. bowel resection, proctocolectomy), whose risks have been reported to be about 46.6% for CD and 15.6% for UC patients 10 years post-diagnosis (Frolkis et al., 2013). In addition, IBD patients are prognosed with an increased risk of colorectal cancer development to an extent of 2.2% on average and 7.0% for long-standing extensive colitis (Beaugerie et al., 2013). Of course, the better surveillance programs in recent years have resulted in decreased malignancy rates of the IBD (Nebbia, Yassin, & Spinelli, 2020). While the exact etiology of the disorder is yet to be uncovered, it is driven by a certain portion of heritability and genetic predisposition. On the other hand, the disease outbreak is often triggered by a number of environmental factors that are contributing to the risk of IBD susceptibility and are linked to urbanization/industrialization, as well as the changes in lifestyle, sanitary standards, nutrition, and indirectly the microbiome (Ananthakrishnan, 2015; Ramos & Papadakis, 2019). Current estimates state a total number of 6.8 million IBD patients (57% females and 43% males) worldwide, and the burden of IBD is still rising from global perspective (Alatab et al., 2020). The incidence rates and trends, however, show substantial regional and national variation. The highest prevalence is found in the United States of America, followed by other industrialized countries in Northern Europe and Australasia. In this industrialized world, the incidence is rather plateauing, while the prevalence may still rise due to the increased life expectancy. In regions with previously moderate incidence, the incidence rates have risen with increasing industrialization. Needless to say, for highly populated countries like India and China, even a moderate increase in incidence has significant effects (Alatab et al., 2020). IBD symptoms persist throughout the patient's life. The low mortality but high destabilization of the disorder in combination with the rather early age of onset results in a high number of years with disability. However, the recent successes in medication development (e.g., introduction of biologicals) for the treatment of autoinflammatory and autoimmune diseases have widened the options for the treatment of the IBD. Within this context, drug discovery and development programs of the last decades have brought quite many candidate compounds for IBD treatment to the pipeline. Still, for a substantial number of patients, this debilitating disease remains far from being controlled in a satisfactory manner. As such, IBD remains associated with unmet medical needs for many patients (Gordon, Mcewan, Maguire, Sugrue, & Puelles, 2015), a disease with a high social and economic burden on governments and healthcare systems (Lichtenstein et al., 2020).

1.1.1 IBD pathophysiology

IBD is an umbrella term used to describe a group of GI tract diseases with common features including the chronic inflammation peculiarity, dysregulation of the immune response and epithelial barrier impairment. Given the variation in disease phenotype, IBD is classified in three main categories: Crohn's disease (CD) and ulcerative colitis (UC) as the main entities, along with a third less prevalent category known as the inflammatory bowel disease, type unclassified (IBDU) (Satsangi, Silverberg, Vermeire, & Colombel, 2006). In terms of pathogenesis, for quite some time, CD has been characterized as a disease with a T helper1 (T_h1) dominated T lymphocyte response, whereas UC has been highlighted as a disorder with T_h17 and regulatory T (T_{reg}) cells as main cellular key players (Zaeem Cader & Kaser, 2013). Naïve T_h cells are primed towards different effector types depending on the inflammatory context and the type of dominant cytokines. As a major cytokine source, dendritic cells (DCs) are drivers of T cell differentiation. While interleukin (IL)-12 and interferon gamma (IFN-ɣ) direct T helper cells towards T_h1 subtype, IL-6 and transforming growth factor-β (TGF-β) induce and IL-23 expands T_h17 cells. The prominent role of T_h17 cells in IBD is related to their secretion of pro- inflammatory cytokines (IL-17A, IL-17F, IL-21, and IL-22) acting on various cell types including epi- and endothelial cells, fibroblasts, and monocytes/macrophages, neutrophils, culminating in inducing inflammation (Geremia, Biancheri, Allan, Corazza, & Di Sabatino, 2014; Wallace, Zheng, Kanazawa, & Shih, 2014). Regulatory T lymphocytes, on the other hand, are the cell type to balance the inflammatory reactions through the secretion of anti-inflammatory mediators such as TGF- β and IL-10.

Figure 1-1 summarizes the alterations of the intestinal mucosal barrier when converting from a healthy to a chronically inflamed state. In the healthy state, the intestinal epithelium as a natural interface to a large microbiota is in an immune-tolerant state, maintained by the constant sampling of some gut luminal material, leading to some degree of immune signals to response trigger, which is then counterbalanced by T_{reg} cells. While the exact causes of the disease remain unclear but seem to be rather diverse (genetic predisposition, environmental stimuli, dysbiosis, aberrant immune response and gut barrier damage) (Ananthakrishnan, 2015), disease manifestation is observed as loss of the tight junction barrier and defects in the protective mucus layer. Combined, these lead to highly increased access of immune stimulatory signals to the gut-associated lymphoid tissue and the resident immune cells. Local immune cells including DCs, macrophages and innate lymphocytes react by secreting a cocktail of pro-inflammatory cytokines for the further recruitment of even more immune cells. Failure to clear

the invasion of antigens leads to an inflammatory cycle, which further damages the intestinal barrier as a result of the continuous immune response. Considering various players within this scenario, the third part of Figure 1-1 shows the targets used by various IBD therapeutics or candidates to interrupt this vicious cycle.

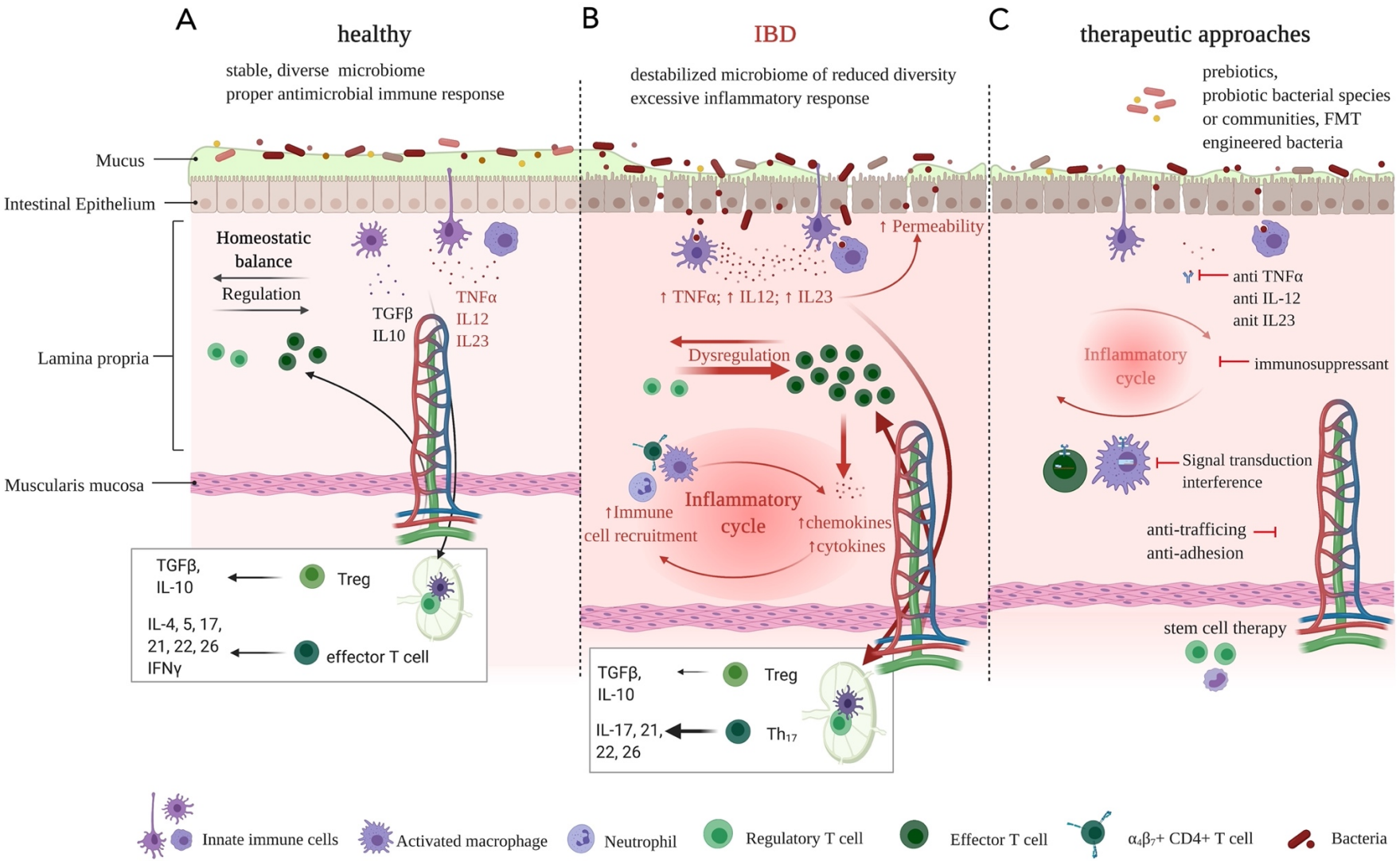

Figure 1-1. Comparison of intestinal homeostasis in healthy intestinal mucosa (A) and IBD conditions with chronic inflammation (B) and therapeutic targets for interference with the inflammatory cycle (C).

1.1.2 Conventional and current medical interventions

As for many other disorders, treatment regimens for IBD management are disease specific. Montreal classification and disease location classifies IBD in terms of its severity into mild, moderate, or severe categories (Satsangi et al., 2006). The first line therapy for mild-moderate active disease is oral mesalazine/5-ASA. Patients unresponsive to this first-line therapy receive oral corticosteroids (e.g., budesonide, prednisone, prednisolone, methylprednisolone) (Kobayashi et al., 2020). Next escalation therapy is oral azathioprine and/or intravenous or subcutaneous TNF-α immunotherapy (e.g., infliximab; adalimumab, golimumab) (Ordás et al., 2012). UC patients with moderate to severe disease activity showing inadequate or lost response, or intolerance to anti-TNF-α immunotherapy receive integrin immunotherapy with Vedolizumab. Severe active UC is immediately treated with first- line therapy of intravenous corticosteroids followed by rescue treatment with TNF-α immunotherapy, or treatment with immunosuppressants (e.g., cyclosporine or tacrolimus). Oral 5- ASA is the standard therapy for maintaining remission. Treatment with azathioprine or anti-TNF-α immunotherapy or vedolizumab is used in case the disease is frequently relapsing.

In CD, 5-ASA is not effective, and therefore, corticosteroids are the first-line therapy. While approximately 20% of the CD patients achieve long standing remission with the first-line treatment, immunosuppressants (e.g., thiopurines or methotrexate) still remain the step-up treatment in case the desired therapeutic effect is not achieved, despite their sub-optimal extent of therapeutic benefit and high rate of adverse events leading to frequent treatment termination (Baumgart & Sandborn, 2012). Fistulizing CD is often managed by a combination of anti-TNF-α antibodies, antibiotics and thiopurines. A milestone in IBD therapy was the introduction of biologicals starting with antibodies against a key pro-inflammatory cytokine in IBD: TNF-α. The first anti-TNF-α antibody approved for IBD was Infliximab, a chimeric antibody with 75% human 25% murine sequence. Further developed antibodies targeting TNF-α were Adalimumab and Golimumab (monoclonal and fully human antibodies), Certolizumab (humanized Fab fragment), and CT-p13 and SB2 as biosimilars of Infliximab. As detailed earlier, the recently approved biologics (e.g., Infliximab, Velodicinab) are used as therapy when first- and second-line therapeutics fail. Despite all such developments, still a certain population of patients remain without satisfying treatment options. TNF inhibitors have more than 1/3 primary nonresponders and an annual risk for loss-of-response of about 13% or 20% in case of

infliximab and adalimumab, respectively. Hence, further expansion of the therapeutic horizons for the management of IBD is highly desirable and consecutively sought after.

1.1.3 Nanotechnology-based drug delivery for inflammatory bowel diseases

Nanotechnology creates a platform, whereby drug targeting to the inflamed tissues can be achieved. When properly formulated, nanoparticles have potential to target the inflamed intestinal not only from the endothelial side upon intravenous injection, but also from the epithelial side following oral or rectal administration (Figure 1-2.). The following sections will focus upon the formulation requirements for nanoparticles to achieve the site-specific drug targeting to the inflamed intestinal mucosa.

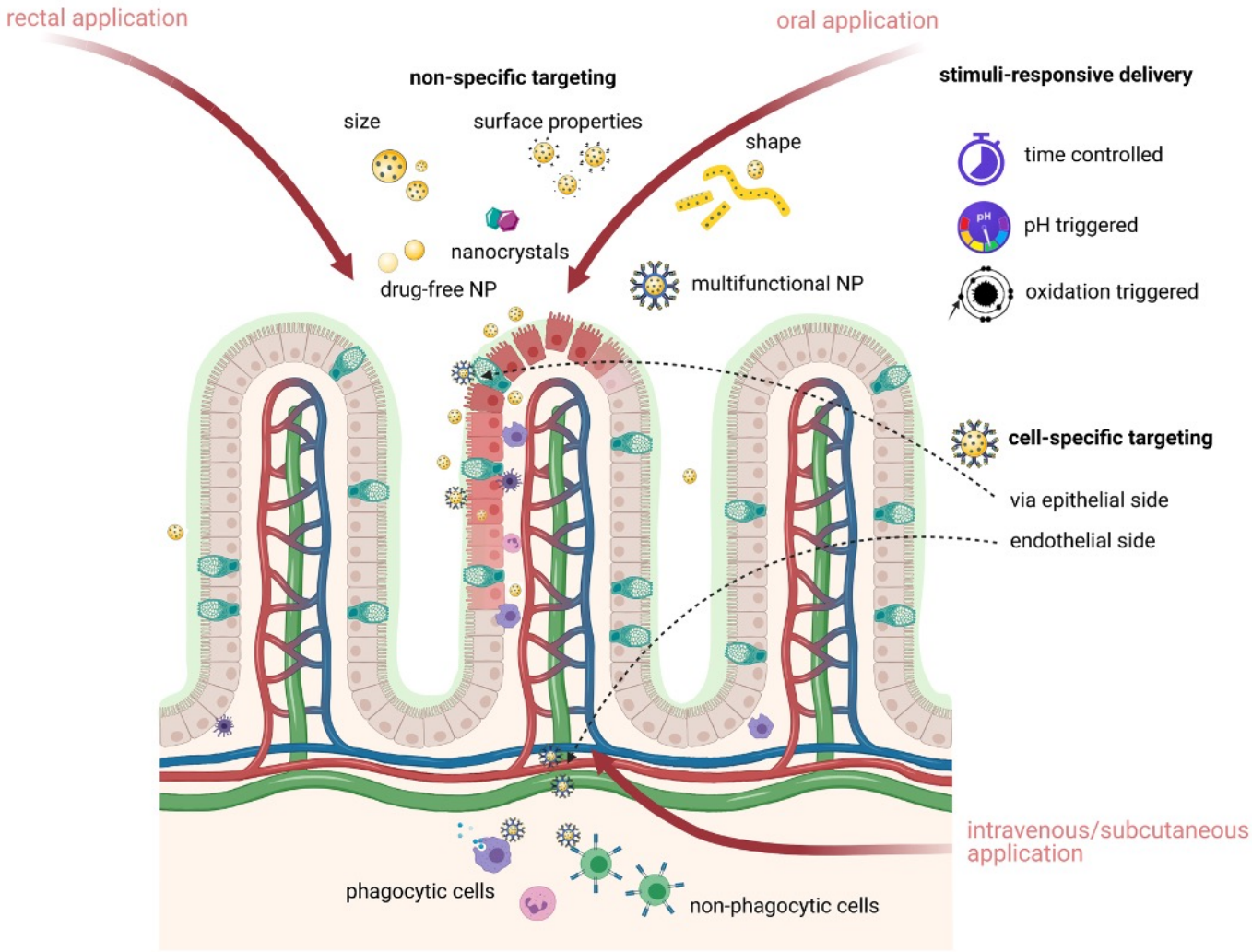

Figure 1-2. Overview of nanotechnology-based targeting strategies for site-specific delivery to the inflamed intestinal mucosa through various routes of administration. Targeting can be either non-specific, based on the physicochemical properties of the nanocarriers, or specific to phagocytic or non-phagocytic immune cells. Nanocarriers can be designed to control drug release over time or in response to specific stimuli.

1.1.3.1 Non-specific NPs: Targeting based on physicochemical properties

1.1.3.1.1 Particle size

Many studies have been performed to determine the effect of nanoparticle size on targeting and accumulation in the intestinal inflammation sites. Most of these benefit from the epithelial enhanced permeation and retention (EPR) effect present in the inflamed sections of the GI tract. The EPR effect results from the defective barrier function of the epithelium at the inflamed intestine or colon, which occurs due to tissue ulceration and the decrease of the amount of the tight junction proteins (Lamprecht, 2015; Zhu, Zeng, Feng, & Wu, 2019). Consequently, the inflamed intestinal tissue can entrap a greater number of particles than the normal healthy tissue. This means that nanoparticles passing through the GI tract will preferably accumulate in the inflamed tissues, as rolling balls end up inside a hole on the road. As this does not require any form of surface modification, it is considered as a form of passive targeting. However, not all particles can fit in the fenestrations of the defective epithelial barrier and enter the intestinal mucosa and the submucosa. This would raise questions about the optimum size of the particles that would allow them to penetrate and accumulate in the inflamed tissue. On top of that, the selected size should allow the particles, before passing through the imperfections in the epithelial barrier, to penetrate and diffuse through the mucin layer that normally covers the surface of the GI epithelium (Date et al., 2018). Although partial depletion of the mucin layer is part of the pathophysiology of the IBD (Sheng, Hasnain, Florin, & McGuckin, 2012), passing through the remaining mucus patches without getting trapped therein is one of the milestones that nanoparticles should achieve to reach the epithelium and deeper intestinal tissue.

Many studies have focused on detecting the effect of the difference in particle size between microparticles and nanoparticles on their retention and accumulation in the inflamed intestinal tissues. Lamprecht et al. were the first to study this phenomenon, where they detected the size dependency of particle deposition in TNBS-induced colitis after oral administration of fluorescent polystyrene particles (0.1, 1, and 10 µm). They reported that the association of the particles to the inflamed tissues was higher than to the non-inflamed areas, with a size dependency in the order of 0. 1 µm>1 µm> 10 µm (Lamprecht, Schäfer, & Lehr, 2001). These results were confirmed by a second study comparing fluorescent Pluronic F127 coated polystyrene micro-and nanoparticles with sizes of 2 µm and 200 nm, respectively (Date et al., 2018). The fluorescent microparticles remained mainly in the lumen of the colon without penetration to the underlying tissues, while nanoparticles penetrated and diffused into colonic

mucosal tissue. Similar results have been reported by another study, where the effect of orally administered curcumin loaded PLGA micro- and nanoparticles with sizes of 1.7 μm and 270 nm were tested on Raw 264.7 macrophages and DSS colitis model. Not only were the nanoparticles more strongly taken up by macrophages compared to their micro-sized counterparts, but they also exerted a higher anti-inflammatory effect in vivo (Ma et al., 2018). On the contrary, Zhou et al. reported higher cellular uptake of 900 nm curcumin loaded nanoparticles by RAW 264.7 macrophages compared to the particles with smaller sizes of 200 and 500 nm. However, further animal studies on TNBS colitis model showed comparable uptake of the nanoparticles of different sizes in the colitis tissue. Yet, 900 nm nanoparticles led to lower amounts of inflammatory mediators both in Raw 264.7 macrophage cells and in vivo (Zhou, Liu, Wang, Li, & Xiao, 2020). The results from in vitro uptake and in vivo accumulation studies suggest that relative to their smaller counterparts, large nanoparticles can have a higher uptake by the phagocytic cells. However, the presence of the mucus layer and the EPR effect, which favor small-sized particles over their larger counterparts, may give the former a better chance to penetrate and accumulate in inflamed intestinal tissues. In this case, although the 900 nm nanoparticles resulted in a better control of the colitis in terms of colon length and histological score, further studies are required to confirm these results, especially as these are against the general conclusion driven from the other studies conducted upon this subject. Most of the reports in the literature point out the superiority of nanoparticles to their micrometric counterparts both in terms of accumulation in inflamed intestinal tissue and the mitigation of IBD.

There have been other studies investigating the effect of particle size within the nanometer range upon the accumulation in the inflamed tissue, and the resultant therapeutic effect. For example, fluorescence imaging of DiI-labeled lipid nanodrops 1 h after administration in murine DSS model demonstrated a higher accumulation of 113 nm nanodrops in the inflamed tissue when compared to their smaller and larger counterparts (54 nm and 183 nm in average, respectively) (Watanabe et al., 2016). These findings suggest that maximum accumulation of the nanoparticles within the inflamed colon and the immune cells requires an optimum particle size of around 100 nm. However, several other studies have results to the contrary. In one study, silver nanoparticles of two different sizes of 213 nm and 95 nm were tested on the DSS colitis model, where the former exhibited higher therapeutic potentials in terms of reducing the disease activity score and tissue MPO activity (Krajewska, Długosz, Sałaga, Banach, & Fichna, 2020). These results were in line with the findings of a recent study

that discussed the difference in the uptake of differently sized nanoparticles (100 and 300 nm) by the immune cells (Youshia, Ali, Stein, & Lamprecht, 2020). Using flow cytometric techniques, 300 nm nanoparticles were shown to have a higher uptake by immune cells compared to their 100 nm counterparts. Other studies focused on particles smaller than 100 nm range to detect if there is a size dependency of their anti-inflammatory effect on IBD. Zhu et al. tested platinum nanoparticles of different sizes of 5, 30, and 70 nm, on the DSS colitis colitis model. After the analysis of disease activity score, histological score, and tight junction proteins, they detected a size dependency in the anti-inflammatory effect of these nanoparticles in the order of 70 nm < 30 nm < 5 nm. However, no size dependency was observed in terms of the particles' ability to reduce the MPO activity, the concentration of the proinflammatory cytokines in the tissue, or the leukocyte count in blood (Zhu et al., 2019).

Based on the results of these previous studies, the general assumption is that the smaller the nanoparticle size is, the higher their accumulation in inflamed intestinal and colonic tissue and the resultant therapeutic effect will be. However, particle size is not the only determining factor within this scenario, but the surface composition of the nanoparticles also plays a crucial role. For example, Zhu et al. performed a similar study to detect the size dependency of tannic acid-coated gold nanoparticles with sizes of 5, 10, 15, 30, and 60 nm, where no significant difference was observed in terms of their therapeutic effect in DSS colitis model (Zhu et al., 2018). A similar observation was reported about tannic acid- coated 122 nm and 294 nm silver nanoparticles, with no significant difference being observed between the two sizes in terms of the reduction of MPO levels or macroscopic score of the inflamed colonic tissue (Siczek et al., 2017). The previous two examples together with the findings of Youshia et al., who demonstrated that nanoparticle surface properties affect their distribution among various inflammatory cells (Youshia et al., 2020), highlight the essentiality of the role of nanoparticle surface properties upon their passive targeting efficiency.

1.1.3.1.2 Surface properties

According to many recent studies, the surface charge of nanoparticles is not less in importance than the particle size for determining the targeting ability to the inflamed intestinal tissue, and the uptake by the resident cells therein. In an interesting study, ethylcellulose nanoparticles of similar sizes (~200 nm), but different in the charge of the decorating surfactant molecules, namely polysorbate 20 (P20), sodium dodecyl sulfate (SDS), and cetyltrimethylammonium bromide (CTAB) were prepared, and the extent of their accumulation

in the inflamed tissue was investigated (Wachsmann, Moulari, Béduneau, Pellequer, & Lamprecht, 2013). In a TNBS colitis model, the percentage of accumulated nanoparticles in the inflamed tissue was the highest for those prepared with the neutral surfactant P20, suggesting that non-charged nanoparticles may have a better chance to penetrate through the mucin layer and to be taken up by the resident inflammatory cells. This enhanced accumulation was translated into a higher ability of the betamethasone-loaded neutral nanoparticles to suppress the production of MPO in the inflamed gut, and hence a better mitigation of the IBD. However, the results of this study might be related not only to the superficial charge of the particles, but also to the structure of the decorating surfactant. Zhu et al. prepared gold nanoparticles of the same size (5 nm) but different in the surface covering moieties (polyvinyl pyrrolidonen (PVP), citrate, or tannic acid). When used to treat the murine DSS colitis, it was found that PVP- and citrate-covered nanoparticles could mitigate colitis more remarkably than tannic acid-covered nanoparticles, as evident from the with former associated lower disease activity index, MPO activity, and histological scores (Zhu et al., 2018). Knowing that that zeta potential of citrate- and tannic acid-covered nanoparticles were close to each other (30.8 ± 2.7 and 37.4 ± 9.6, respectively) and very different from the PVP-covered nanoparticles (0.2 ± 0.7), a crucial effect of the nature of the surface covering moiety can be affirmed. Therefore, to detect the pure effect of particle surface charge, Iqbal et al. prepared lipid-associated PEG5K-b- PLGA10K nanoparticles of similar sizes (105.5–127.5 nm) loaded with siRNA. By fine-tuning the coupling reaction of the surface lipids with succinic anhydride, they were able to produce nanoparticles of the same composition and particle size, differing only in the surface charge (cationic, neutral, and anionic). The results showed that cationic nanoparticles were more powerful than their neutral and anionic counterparts in the reduction of TNF-α levels, and the weight loss, and improving the histological score due to their significantly higher accumulation in the inflamed regions of the colon (Iqbal et al., 2018a). The results of this study are also in line with a recent report, showing that cationic nanoparticles had a higher uptake by the inflammatory cells (Youshia et al., 2020).

Based on the hitherto performed investigations, it can be concluded that surface properties, including but not limited to surface charge, affect the targeting and accumulation of the nanoparticles in inflamed intestinal tissues. Based on this concept, the development of mucus-penetrating particles (MPP) was proposed for improved epithelium targeting. One study showed that dense coating of polystyrene nanoparticles with polyethylene glycol (PEG) moieties endows them mucus penetrating properties and allows them to accumulate more in the

intestinal tissues. Uncoated nanoparticles, on the contrary, were mostly entrapped in the mucus layer of the gut (Maisel, Ensign, Reddy, Cone, & Hanes, 2015). Another research group showed that the cross-linking between polyacrylic acid (PAA) and polyallylamine (PAM) results in the formation of neutral MPPs with higher mucus penetrability ($2.81 \pm 0.35 \times 10^{-6}$) compared to pure PAA and PAM nanoparticles ($1.51 \pm 0.52 \times 10^{-6}$ and $1.15 \pm 0.23 \times 10^{-6}$, respectively) (Laffleur et al., 2014). Date et al. compared 200 nm nanoparticles covered with poloxamer F127 and PVP and showed that the former had higher accumulation in the colonic tissue, while the latter remained in the lumen of the gut (Date et al., 2018). These studies indicate that not all long-chain neutral polymers will impart nanoparticles mucus penetrating properties, and thus increase their accumulation in the colonic tissue. Therefore, further studies are required to determine which types of neutral polymers can best serve this purpose.

1.1.3.1.3 Particle morphology

Considering the significance of the size and surface properties of the nanoparticles for their targeting ability within the context of IBD drug delivery, it is plausible to assume that particle morphology might also exert an impact. In addition, morphology might also impact the mucus penetrability of the particles as well as their uptake and distribution pattern among various types of intestinal and colonic resident inflammatory cells. Within the context of the MPPs, several studies have strived to investigate the impact of particle shape. In one work, the mucus penetrability of small nanosphere and short nanotube peptosomes, prepared through the self-assembly of amphiphilic α-lactalbumin, was compared. The results showed that short nanotubes had enhanced penetrating properties through the mucus layer, reaching thereby the underlying epithelium quicker than their spheric counterparts. When loaded with curcumin, nanotubes, unlike the nanospheres, resulted in the optimum management of the DSS colitis. The high permeability of short nanotubes through mucus was demonstrated through coarse-grained molecular dynamics simulations, which indicated that short nanotubes were able to jiggle and pass across the pores of the mucus network (Bao et al., 2020a). Similar results were obtained in another study, which demonstrated calcium phosphate and mesoporous silica nanorods had higher penetration through the mucus and longer retention time in the gut compared to the nanospheres of the same composition (Yu et al., 2016). These reports are also in line with another work that was performed to determine the effect of the difference in particle shape on the cellular uptake of the nanoparticles, where polystyrene nanorods were taken up more strongly than spherical and disk-shaped nanoparticles (Barua et al., 2013). Hence,

nanorods seem to be the best morphology to be used for the mitigation of the IBD. This could be due to the non-spherical dimensions (high aspect ratio) that nanorods have. While a nanosphere has a diameter that determines the size of the particle, nanorods can be made rather thin but long enough to penetrate through the mucus layer and through the defective epithelial lining through their rotational motions. As a result, the thickness of the nanorod may be smaller in dimension than the defects in the lining of the inflamed colon (Yu et al., 2016). Of course, not all nanoparticles can be produced as rods, and the morphology is usually determined by the chemical composition of the nanoparticles and the production techniques. Furthermore, more in-depth research is required to optimize the trifecta of nanoparticle size, surface properties and morphology to maximize the targeting ability of the carrier within the context of IBD treatment.

1.1.3.1.4 Carrier- and drug-free nanoparticles

Being rather small molecules, most drugs are absorbed transcellularly, either by simple diffusion and/or binding to a specific transporter, or paracellularly by passage through the tight spaces between the epithelial cells lining the mucosa to the underlying cells and beyond (Yun, Cho, & Park, 2013). However, nanoparticles are too large to pass through the intact colonic mucosa but may use the opened tight junctions or cellular voids in the inflamed epithelium resulting in selective accumulation of the drug in the inflamed tissue. In addition to the targeted delivery of the drug, another challenge within the context of colitis treatment is the delivery of adequate amounts of the drug to exert its pharmacological effect (De Jong & Borm, 2008). Although the previously debated factors, namely particle size, surface properties, and particle morphology could affect the targeting and accumulation in inflamed tissue, the amount of the drug loaded within such particles should be maximized to observe an efficient therapeutic function. Carrier-free nanoparticles e.g., nanocrystals and nanosuspensions, mainly consist of nanosized drug particles suspended in a stabilizer solution (e.g., surfactant and/or polymer), and are associated with the highest amount of drug per particle compared to other types of nanocarriers (Y. Wang, Zheng, Zhang, Wang, & Zhang, 2013). As the nanometric size of such systems enables their passive accumulation within the inflamed intestinal tissue, and considering the high drug load associated therewith, it comes as no surprise that carrier-free nanoparticles have been already investigated for the IBD treatment. In one study, budesonide nanosuspension was prepared and tested in the TNBS colitis model, where it led to a significant improvement of the inflammatory condition by decreasing the number of inflammatory macrophages and the inflammatory cytokine IL-1β, leading to the improvement of the disease

activity index and the histopathological score (Date et al., 2018). In another work, curcumin nanocrystals were covered by chitosan and alginate shells to protect the nanocrystals from the premature release of the drug. The results suggested higher accumulation of the nanocrystals in the inflamed colonic tissue compared to the healthy regions, and enhanced anti-inflammatory effect evident from the reduction of tissue MPO levels (Oshi et al., 2020). Although the concept of nanocrystals and nanosuspensions is very promising, limited studies have been performed in the field of colitis management. This, of course, inspires further research regarding the tuning of the shape, size, and surface properties of these carrier-free formulations to maximize their efficiency for drug targeting to colitis.

It is well established that given their nanometric size, nanoparticles can interact with the cells of the immunity, resulting either in the activation or the inhibition of the immune response. While the exact nature of such complex interplays is yet to be uncovered, the material used for nanoparticle formulation has been shown to play a significant role. Chitosan, for example, has been shown to reduce the rate of mortality in the mice suffering from DSS colitis. It also accounted for a better control of the disease by reducing the NF-κB, TNF-α, and IL-6 in the acute and chronic colitis model (Yousef, Pichyangkura, Soodvilai, Chatsudthipong, & Muanprasat, 2012). Comparable results have been reported in another study, which showed that chitosan possesses anti-inflammatory properties independent from its degree of deacetylation and molecular weight (Jhundoo et al., 2020). Other nanoparticles have been shown to exert an anti-inflammatory effect within the context of colitis treatment. Moulari et al. demonstrated that drug-free nanoparticles of ammonio methacrylate copolymers could significantly mitigate TNBS colitis in mice. Even in the absence of a drug load, the nanoparticles were able to decrease the inflammation state of the colon by reducing the production of inflammatory mediators and cytokines, including TNF-α, IL-6, IL-1β, and IL-12, and the level of alkaline phosphatase and MPO. This was attributed to the ability of the nanoparticles to deplete colonic resident immune cells by virtue of their cationic charge, improve the barrier function, and scavenge and neutralize the endotoxin in the gut (Moulari et al., 2020). Silk fibroin nanoparticles have been also shown to reduce the expression of inflammatory mediator IL-1β, the colonic oxidative stress, and the intestinal neutrophil infiltration, alleviating thereby the TNBS colitis in mice. Besides, the particles were able to restore the intestinal wall integrity as evident from the higher expression of the associated markers (TFF-3, mucins and villin) (Rodriguez-Nogales et al., 2016). Other studies prepared metal nanoparticles e.g., platinum, gold, and silver, which all exerted remarkable anti-inflammatory effects, and were able to

significantly alleviate IBD as discussed previously (Zhu et al., 2018, 2019). Taken together, the results of the hitherto performed studies using carrier-free and drug-free nanoparticles are very encouraging, as they highlight the possibility of designing of delivery systems, in which both the drug and the carrier can exert a pharmacological action and would potentially perform superior to the normal inert-carrier nanoparticles within the context of IBD treatment. However, whether this combination can result in a synergistic effect is yet to be investigated.

1.1.3.2 Stimuli-responsive nanoparticles: drug release depends on (patho-) physiological triggers

1.1.3.2.1 Transit time and pH of the GI tract

Many of the hitherto developed targeting methods for IBD therapeutics either physically delay the drug release from the nanoparticles until they reach the colon, or else protect the nanoparticles with a polymer that dissolves under the effect of colonic pH or microbial enzymatic activities. Regardless of the used strategy, the goal here is to inhibit the premature release of the drug in the upper part of the GI tract. While these strategies are suited for colonic drug delivery in healthy individuals, they are not as reliable in IBD patients, as the disease might be associated with a change in the colonic pH and the microbiome composition, and the diarrhea may affect the GI transit time, resulting in unexpected early or incomplete release of the cargo from such nanoparticles (Wachsmann & Lamprecht, 2012; Youshia & Lamprecht, 2015; M. Zhang & Merlin, 2018). Therefore, we will focus on other, more IBD-oriented stimuli-responsive strategies for colon targeting.

1.1.3.2.2 Oxidative stress

Although the pH and the transit time may be affected by the disease state and the symptoms associated with IBD, oxidative stress is a hallmark of inflammation at any site of the GI tract. Therefore, the design of nanoparticles that can release their cargo only at the sites with increased ROS activity is a very promising colitis targeting technique. Most of the strategies used to this end are based on the self-assembly of oxidative stress-responsive polymers, which disassemble upon exposure to oxidative enzymes or ROS, and release the drug loaded therein. The combination of the nanometric particle size and the site-specific release renders such particles good candidates for the passive targeting of the inflamed regions of the gut. An interesting study demonstrated that the use of budesonide and tempol loaded in ROS-responsive aromatized thioketal nanoparticles on the inflammatory macrophages stimulated with phorbol-

12-myristate-13-acetate (PMA) resulted in the release of more than 98% of the drug load. These nanoparticles were then tested on the DSS colitis model and were associated with a higher accumulation of the loaded drugs in the inflamed, rather than the healthy, colonic tissues. This was attributed to the passive targeting due to the small size (100–120 nm) of the nanoparticles, along with the site-specific release of the drug at the inflamed parts of the colon (S. Li, Xie, Li, Zou, & Zhang, 2019). In a different study, ROS- responsive carriers were prepared via the derivatization of β cyclodextrin (OxbCD) and loaded with a pro-resolving annexin A1-mimetic peptide Ac2-26 by nanoprecipitation and self- assembly. Having tested the particles on the DSS colitis model, higher amounts of Ac2-26 were found in inflamed parts of the colon. The site-specific release and the high accumulation of Ac2- 26in was translated into a decrease in the pro-inflammatory mediators and inflammatory cell infiltration in the colonic tissue. Accordingly, the symptoms of inflammation alleviated, which facilitated intestinal mucosal healing (C. Li et al., 2019). Although the findings of these experiments are promising within the context of the IBD treatment, their performance in severe pathological conditions of colitis is yet to be explored, as excessive mucus production may prevent them from reaching the inflamed mucosa, or heavy diarrhea may prematurely expel them from the colon.

1.1.3.3 Cell-specific nanoparticles: targeting a specific immune cell or inflammatory mediators

All previously mentioned strategies can be considered as examples of passive targeting, where the preferential distribution or release of the nanoparticles occurs passively at the site of inflammation. In this section, the active targeting of the nanoparticles to specific cells or to the inflamed tissue will be discussed. Here, targeting may occur by virtue of the presence of ligands on the nanoparticle surface that bind to specific receptors on the target cells, or it might originate from the preferable uptake of the carrier material by specific cell types. The most prominent cellular targets for reversing the inflammation are the inflammatory cells e.g., macrophages, neutrophils, DCs, and lymphocytes. Inflamed epithelial cells and the released inflammatory mediators are also good targets for nanoparticles to bind to, so that their therapeutic cargo can be released in the relevant site of action. Many of these cell- targeted nanoparticles have been extensively reviewed elsewhere (Yang & Merlin, 2019). Therefore, we will only touch lightly upon the most recent studies that have dealt with the development of actively targeted nanoparticles for colitis treatment.

1.1.3.3.1 Phagocytic cells

Macrophages are the first line of defense in the intestinal tissue; they constantly scan the area for any sign of infection or injury. In case of finding such cues, they respond by phagocytosing the invader in case of an infection, followed by the release of a variety of inflammatory mediators and cytokines that promote the infiltration of other inflammatory cells to the area (Cucchiara, Stronati, & Aloi, 2012). Therefore, active targeting of macrophages using drug-loaded nanoparticles would be of great value in breaking the inflammation cycle. CD44 is a transmembrane glycoprotein present on inflammatory macrophages, assisting in macrophage cell proliferation and migration (Rios de la Rosa, Tirella, Gennari, Stratford, & Tirelli, 2017). A recent study used chondroitin sulfate as a targeting moiety that can preferentially bind to CD44 on macrophages to enhance the uptake of curcumin-loaded PLGA nanoparticles decorated therewith. Cell experiments confirmed enhanced nanoparticle uptake by macrophages and a remarkable decrease in the secretion of the pro-inflammatory cytokines (X. Zhang et al., 2019). A different study benefited from the preferential uptake of lipid nanoparticles by macrophages (B. Wang et al., 2014). Here, lipid nanoparticles derived from the natural fruit Lycium barbarum were shown to suppress the production of the proinflammatory cytokines TNF-α and IL-12, and promote the secretion of the anti-inflammatory cytokine IL-10 (S. Li et al., 2019). Similarly, a recent study exploited ginger lipid-derived nanoparticles for the targeting of CD98-siRNA to macrophages (M. Zhang et al., 2016). Mannose receptor also provides an interesting target on the activated macrophages abundantly present in the colonic tissue. Sun et al. prepared a nanocomposites through binding inulin-loaded and mannose-coupled nanoparticles to carbon dots. In vitro cell experiments showed the preferential uptake of these nanoparticles by activated macrophages, while the in vivo experiments also revealed their preferential accumulation in the inflamed tissue (Sun, Arif, Chi, Li, & Liu, 2021). Although macrophage targeting has presented a very efficient technique to target the inflamed sites of the GI tract, systemic administration of macrophage targeted nanoparticles might result in undesirable side effects or compromised immune response given the omnipresent nature of these cells (Lopez-Yrigoyen, Cassetta, & Pollard, 2021). Therefore, such nanoparticles are preferably applied for epithelial, rather than endothelial, targeting of the intestinal and colonic macrophages.

Together with macrophages and epithelial cells, DCs produce an initial, immediate response to any pathogen or tissue injury (Cucchiara et al., 2012). Therefore, active targeting of the nanoparticles towards the intestinal and colonic resident DCs is highly desirable. A

previous study suggested mannose-grafted nanoparticles to target DCs as well as macrophages (Coco et al., 2013). Another study demonstrated the significant effect of edible plant-derived nanoparticles in the prevention of the IBD by virtue of the DC deactivation. Using broccoli-derived nanoparticles in a colitis model, it was found that these nanoparticles activate the adenosine monophosphate-activated protein kinase, which in turn prevents DC activation and aids in the creation of a tolerant state (Deng et al., 2017). As the role of DCs in the inflammatory cascade is limited to the presentation of various antigens to lymphocytes (Sallusto & Lanzavecchia, 2002), their targeting may not produce a huge therapeutic benefit and is often reserved for the prevention of the colitis recurrence.

Recent studies, however, have gone for a different perspective based on targeting the abundantly present inflammatory mediators in the inflamed colonic tissues, rather than certain subsets of inflammatory cells. Wang et al. used supramolecular nanoparticles containing the anti-TNF-α monoclonal antibody infliximab for the treatment of DSS colitis model, where a higher accumulation of the particles in inflamed colonic tissue and the low systemic concentration of the drug was achieved. The particles also brought about significant anti-inflammatory effects as evident from the improved weight and colon length, and the suppressed secretion of various pro-inflammatory mediators (TNF-α, IL-1β, and IL-6) (X. Wang et al., 2020).

In addition, when studying the surface coating of nanoparticles with a targeting ligand, the possible loading scenarios after administration of these nanoparticles should be considered. For example, charged nanoparticles interact and bind with opsonizing proteins or with GIT proteins when administered intravenously or orally, respectively. Such corona formation can lead to a loss of targeting ability of the nanoparticle and nonspecific binding of the nanoparticles, leading to release of the drug in the reticuloendothelial system (RES) organs such as liver and spleen, which may lead to systemic side effects or unintended inhibition of immunity in non-inflamed colon tissue, respectively (Aggarwal, Hall, McLeland, Dobrovolskaia, & McNeil, 2009; T. Zhang, Zhu, Lu, Qian, & Peng, 2021). Therefore, novel nanoparticles should be examined ex vivo in physiological fluids to predict the possible incompatibilities that may occur in vivo (R. Liu, Kay, Jiang, & Chen, 2009).

1.1.3.3.2 Non-phagocytic cells

The ability of the immune system to protect the body against external invaders does not only depend on the phagocytic activity of the innate immunity, but also on the function of the

non- phagocytic cells of the adaptive immunity such as lymphocytes. These cells are often recruited to the site of inflammation and release antibodies that help destroy the predator, or else neutralize the toxins secreted thereby.

Lymphocytes home in the inflammation sites by binding to specific adhesion molecules, whose expression is upregulated on the luminal side of the endothelial cells under the effect of various abundantly secreted pro-inflammatory cytokines. Many studies have been dedicated to mimicking the homing ability of the lymphocytes, and other leukocytes, to the site of inflammation through binding to these adhesion molecules. For example, the MAdCAM-1 is an endothelial receptor that can bind to α4β7 integrin, which is overexpressed by a subset of T-lymphocytes. Therefore, some researchers designed leukocyte-like carriers expressing the α4β7 integrin and tested for their ability to target the inflamed sites of the colon. Systemic administration of these particles could successfully reduce the inflammation as well as the number of immune cells infiltrated into the colonic tissue (Corbo et al., 2017). Other studies used the same concept, but with different ligand-receptor combinations. In several studies, biodegradable polymeric nanoparticles carrying superficial ligands for the upregulated endothelial adhesion molecules, e.g., P-selectin, E-selectin, intercellular adhesion molecule, and vascular cell adhesion molecule 1 were prepared, which showed strong attachment to endothelial cells compared to plain nanoparticles (Eniola & Hammer, 2005; Eniola, Rodgers, & Hammer, 2002; Sakhalkar et al., 2003). These nanoparticles should be thus further investigated in terms of their safety and efficiency within the context of IBD treatment.

1.1.3.4 Multifunctional NPs

The improvement of our knowledge in respect with the factors that can effectively improve the targetability of nanoparticles to the inflamed intestinal tissues, along with the growth of our understanding regarding the limitations associated with each, has inspired the development of multifunctional nanoparticles, whereby the limitation of each approach can be made up for the strength of the others. For example, ECM coatings are often applied to nanoparticles to give them the ability to target specific cells, such as macrophages or lymphocytes. However, they do not impart responsive release properties to the nanoparticles. Therefore, although these NPs can be selectively targeted to inflamed tissue, the cargo may suffer from premature or off-target release. On the other hand, polymer-coated nanoparticles have the inherent ability to selectively target inflamed colon tissue at these inflamed sites due to their small particle size and/or trigger-dependent release of the drug. However, due to the

non-specific binding and limited selectivity of polymeric nanoparticles, they may also reach unintended sites in the colon. Therefore, it is best to prepare polymeric ECM-coated nanoparticles or multifunctional nanoparticles. Decoration of polymeric nanoparticles with ECM allows them to passively reach inflamed colon tissue and actively attach to target cells, which can then be selectively treated with their cargo (Frickenstein et al., 2021). Multifunctional nanoparticles make use of more than one targeting technique, or else exploit the therapeutic potentials of both the carrier and the drug. Formulation of nanoparticles using various polymers with different properties (e.g., stimuli- responsiveness), and their decoration with various active targeting moieties increases the probability that the nanoparticles accumulate in certain cells of the inflamed tissue and exert their pharmacological action in the right place. For example, Gou et al. have prepared curcumin-loaded nanoparticles composed of silk fibroin and chondroitin sulfate, combining various techniques discussed earlier. While such nanoparticles preferably accumulate in the inflamed colonic tissue by virtue of their nanometric size, chondroitin sulfate helps target the colonic macrophages, where both silk fibroin, the carrier, and curcumin, the active, will exert their anti-inflammatory effects (Gou et al., 2019). In a recent study, low molecular weight heparin (LMWH)-loaded nanoparticles were prepared from sodium alginate and trimethyl chitosan (TMC). The aim was for alginate to protect the nanoparticles from premature drug release in the gut, until they reached the colon, where the alkaline pH allows for alginate to dissolve, exposing the underlying chitosan nanoparticle (Yan et al., 2020). As discussed earlier, chitosan is well-known for its mucoadhesive and macrophage targeting abilities (Yang & Merlin, 2019), and simultaneously possesses anti-inflammatory properties. Combined, all these, along with the nanometric size of the particles, allow for chitosan to target the macrophages in the inflamed colonic tissue with a prolonged residence time, where together with the released LMWH, they can exert an additive, if not a synergistic, therapeutic effect. The introduced examples represent only the beginning of a new era, where a new generation of nanoparticles are designed to safely pass through the GI tract, penetrate the mucus layer, target the inflamed sites of the intestine or the colon, remain longer in the site of action, and exert an additive, or even a synergistic, therapeutic effect associated with both the drug and the carrier. However, the development of such nanoparticles is often complicated within the context of industrial production and clinical translation, and numerous aspects must be explored before they can be considered as a viable clinically approved alternative to the conventional therapies for the management of IBD.

1.2 Drug Nanocrystals (NCs)

The potential for developing nanoformulations for the treatment of IBD has been extensively studied. Drug is usually formulated in nanoparticles (NPs) to enhance its tissue penetration and improve its ability to reach inflamed better than non-inflamed tissues (F. Chen, Liu, Xiong, & Xu, 2021). NPs are mainly composed of the drug substance and an inert carrier, such as a polymer or lipid. Consequently, many varieties of nanoformulations have been exploited for drug delivery, comprising nanoemulsions, liposomes, nanocomplexes, and polymeric nanoparticles (Lu, Zhang, Wang, & Chen, 2021). Though the carrier is typically selected to augment the physicochemical properties of the NPs to serve the intended purpose, they form the bulk of NPs and suffer from low stability and early leakage of the active ingredient. A subcategory of these NPs consists of nanometric drug particles coated by a suitable stabilizer and/or surfactant and are known as nanocrystals (NCs). NCs consist mainly of the active ingredient, without any carrier component unlike polymeric/lipidic nanoparticles, which imparts them maximum drug loading and relatively higher stability than all other NPs (Gao et al., 2012).

NCs can be produced by either top-down or bottom-up methods, depending on the size of the starting material. The former involves the size reduction of large solid drug particles by mechanical methods such as high-pressure homogenization, wet milling, and microfluidization, while the latter is based on the assembly of drug molecules into NCs. This bottom-up assembly of NCs is performed either by solvent evaporation, e.g., nanoprecipitation, or by antisolvent methods, e.g., liquid antisolvent (Chogale, Ghodake, & Patravale, 2016). Top-down methods, unlike bottom-up methods, do not use harmful organic solvents since the active ingredient is dispersed but not dissolved in water. Therefore, top-down methods can be regarded as eco-friendly.

Wet media milling is one of the most used top-down methods for manufacturing NCs (Malamatari, Taylor, Malamataris, Douroumis, & Kachrimanis, 2018). It involves the application of mechanical energy to exert stress on particles, resulting in their fracture and deformation. Fracture occurs through crack formation and propagation (Peltonen & Hirvonen, 2010). Two types of wet mills have been proposed for the application of this stress, the high shear homogenizer and wet milling. The milling medium in wet milling is typically beads of a

dense, hard material (stainless steel, alumina, titanium, yttrium-stabilized zirconia, or glass), which are used to crush the coarse drug particles and disperse them in the stabilizer solution.

Therefore, many process factors can affect the particle size of NCs produced by wet milling, such as the diameter of the milling beads, the weight ratio of drug to beads, the concentration and volume of the stabilizer solution, and the milling time (Date et al., 2018). However, the optimum level of each factor depends on trial and error. Therefore, the objective of chapter 3 was to evaluate the effects of these factors on the particle size of NCs produced by wet milling in order to select the optimum level of each factor to produce the desired particle size. Understanding the effects of these factors will allow further fine-tuning to prepare drugs NCs of specific particle size, as will be discussed in chapters 4 and 5.

1.3 Rivaroxaban (RX)

IBD is usually accompanied by a hypercoagulable state and thrombus formation within the inflamed gut and in extra-intestinal tissues (Vrij, Rijken, Van Wersch, & Stockbrügger, 2003). Studies have shown that the imbalance between the levels of pro-coagulant substances such as thrombin and the levels of endogenous anticoagulant substances e.g., activated protein C is responsible for the spread of inflammation. There is an interdependence between inflammation and coagulation, creating a vicious cycle in which each process reinforces and drives the other. For instance, platelets and many components involved in coagulation, e.g., thrombin and tissue factor, have been found to increase inflammation when activated at sites of thrombus formation (Esmon, 2005). However, anticoagulants, e.g., protein C and heparin, show anti-inflammatory effects. Moreover, many inflammatory mediators and their signaling pathways impair the fibrinolytic system and reduce natural anticoagulant activity, leading to a hypercoagulable state (Yoshida & Granger, 2009).

This interdependence of inflammation and coagulation has allowed the development of novel therapeutic strategies for IBD dependent on inhibition of the coagulation pathway. For example, inhibition of platelet activation with clopidogrel has resulted in a significant decrease in MPO activity in intestinal tissues and improvement in clinical symptoms of the disease (Patel, Rachchh, & Jadav, 2012). In addition, the use of direct thrombin inhibitors, e.g., low-molecular-weight heparin and argatroban, resulted in macroscopic and histologic improvement of the inflamed colon and reduction of MPO activity therein (Lean et al., 2015; Onomura et al., 2000; Yazeji et al., 2017). Moreover, factor Xa, which is involved in coagulation cascade, is well-known for its pro-inflammatory effect through activation of proteinase activated receptor

2 (PAR-2) (Borensztajn, Peppelenbosch, & Spek, 2009). PAR-2 is expressed all over the gastrointestinal tract on various cell types implicated in immune and inflammatory responses, modulation of endothelial-leukocyte interactions, and regulation of secretion of inflammatory mediators (Cenac et al., 2002). Therefore, rivaroxaban (RX), a factor Xa inhibitor, would be a promising candidate in colitis therapy. However, nonspecific administration of RX to inflamed and noninflamed colon tissue could lead to an unintended anticoagulation effect in the colon, which could exacerbate preexisting GIT bleeding associated with IBD. Therefore, selective administration of RX to inflamed tissue sites will be of great benefit to break the vicious cycle of inflammation and coagulation at these sites and sparing noninflamed sites from the anticoagulant effect.

1.4 Hemin

New classes of drugs such as hemin have been discovered that reverse inflammatory mechanisms and improve the treatment of IBD. Heme, or hemin, is a part of hemoglobin that is responsible for oxygen transport in the body. However, the presence of heme in tissues signals tissue injury and leakage of blood from capillaries. Therefore, heme is considered one of the damage- or danger-associated molecular patterns (DAMPs) (Gladwin & Ofori-Acquah, 2014; Pradhan, Vijayan, Gueler, & Immenschuh, 2020). On the other hand, hemin solution was tested in a model of experimental colitis, where it inhibited the expression of a subset of genes encoding inflammatory mediators such as IL6 and IL1a, that are induced by lipopolysaccharides (Kayama et al., 2018). In addition, hemin is an inducer of heme oxygenase-1 (HO-1), an important enzyme in heme metabolism, which produces antioxidant and anti-inflammatory products such as carbon monoxide and biliverdin (Mateus, Rocha, Mota-Filipe, Sepodes, & Pinto, 2018; Zhong et al., 2010). Therefore, hemin lowers the levels of many inflammatory markers in colon tissue by modulating macrophage activity in the gut and producing endogenous antioxidant and anti-inflammatory products. In this context, hemin acts as a double-edged sword, and formulating it as NCs that would passively target inflamed tissue allowing it to exert its anti-inflammatory effects while sparing healthy tissue from its inflammatory effects.

Scope of work

Inflammatory bowel diseases (IBD) are a group of inflammatory chronic diseases that comprise ulcerative colitis (UC) and Crohn's disease (CD). They are hallmarked by impaired integrity of the epithelial barrier of the intestinal mucosa because of progressive inflammation, ulceration of the intestine, and activation of the immune system. Activation of the intestinal immune system leads to the release of proinflammatory cytokines, e.g., prostaglandins (PG), interleukin-1β (IL-1β), leukotrienes (LT), and tumor necrosis factor (TNF-α). As a result, clinical symptoms typically include abdominal pain, weight loss, bloody diarrhea, and anemia.

Drug treatment of IBD was and still dependent on the reduction of the inflammation state and treatment of the symptoms. For decades mesalazine/5-ASA was used for mild or moderate colitis, and corticosteroids or immunosuppressants were used in severe cases. However, the continuing research and understanding of the pathophysiological mechanisms of IBD led to the development of more specific drugs that can break the vicious cycle of inflammation by neutralization of one of the inflammatory mediators, e.g., anti-TNF-α, anti-IL12/23, and anti-IL13. A new category of drugs is also under development, which depends either on the remodeling of the inflammation microenvironment by the inhibition of indirect proinflammatory factors, e.g., low molecular weight Heparin and anticoagulants, or the release of anti-inflammatory enzymes as a byproduct of their metabolism in the tissue (e.g., hemin).

Moreover, in managing IBD, the oral and systemic delivery of drugs, is challenging because these molecules can lead to serious adverse effects at off-target positions. Therefore, it is highly desirable to achieve higher bioavailability at the site of action and reduce systemic exposure. To achieve this goal, an efficient delivery vehicle is required that is capable of ensuring adequate stability of the drug until it reaches the target site, releasing the drug, ideally, in a timely manner, and retaining the drug load in the target site to ensure its efficient function. Nanoparticles (NPs) have been widely used for the selective delivery of drugs to inflamed colonic tissue as their nanometric particle size allows them to accumulate in these sites via an enhanced permeation and retention (EPR) effect. However, drug-loaded NPs made of polymers or lipids experience low drug loading because the bulk of nanoparticles are composed of their inert carrier.

Carrier-free nanoparticles e.g., nanocrystals, consist mainly of nanoscale drug particles coated with a polymer and/or surfactant, and contain the highest amount of drug per particle compared to other types of nanocarriers. As their nanometric size facilitates their passive targeting to the inflamed intestinal tissue, their high drug load can ensure the arrival of

therapeutic amount of drug at that target. Although the concept of nanocrystals and nanosuspensions is very promising, few studies have been conducted in the field of colitis treatment. Moreover, the effects of particle size, surface charge, and particle shape of NPs on their efficacy in IBD have been extensively studied. However, limited studies have been conducted to evaluate these effects on the efficacy of NCs. This, of course, encourages further research to evaluate these effects on carrier-free formulations to maximize their efficiency for targeted drug delivery in colitis.

NCs can be prepared by top-down or bottom-up methods, based on the size of starting material. Top-down methods, unlike bottom-up methods, are safer and environmentally acceptable as they don't depend on the usage of organic solvents. However, different experimental factors must be optimized to reach the desired particle size of the NCs using these types of methods, as will be discussed in **Chapter 3**.

In parallel to evaluating the effect of particle size, charge and morphology on the efficacy of NCs formulations, we also aimed to investigate new classes of drugs in order to enhance their effects in IBD treatment. Since the use of heparin in the treatment of IBD has been previously studied, we wanted to evaluate the efficacy of another anti-coagulant for the treatment of IBD by formulating it as NCs. Therefore, we prepared rivaroxaban NCs with different particle sizes (nano- and microcrystals) and surface charges (anionic, cationic, and nonionic nanocrystals) and then studied their anti-inflammatory effects in vitro using macrophage cell lines and in vivo using the TNBS colitis model, as will be described in **Chapter 4**.

As the shape of rivaroxaban NCs was irregular, it could not be determined whether NCs with a different shape from polymeric NPs are able to passively deliver the drug to the target as NPs do or to enhance the anti-inflammatory effect of the drug as compared with solution. Therefore, Hemin was selected for the second study because it generates nanoneedles upon milling. Accordingly, Hemin NCs were prepared, characterized, and then also tested for their anti-inflammatory activity in macrophage cells and in the TNBS-induced in vivo colitis model, as discussed in **Chapter 5**.

The ultimate goal of this work was to investigate the ability of nanocrystal technology to enhance the delivery of drugs to inflamed colon tissue in order to develop a better management of IBD than the current conventional therapeutic approaches.

2 Materials and methods

2.1 Materials

Lumogen® yellow S 0790 was a gift from BASF (Ludwigshafen, Gemany), rivaroxaban and hemin were purchased from Sigma-Aldrich (St. Louis, USA). Sodium lauryl sulfate (SDS) and Kolliphor® P 188 (poloxamer 188) were obtained from VWR (Darmstadt, Germany). Cetyltrimethylammonium bromide (CTAB) was from Sigma-Aldrich (Taufkirchen, Germany). Hydroxypropylmethylcellulose E6 (HPMC) was a gift from JRS pharma GmbH (Rosenberg, Germany) and Zirconium beads were from VMA-Getzmann GmbH (Reichshof, Germany). Dulbecco's minimum essential medium (DMEM), penicillin G, fetal bovine serum (FBS), streptomycin, and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, USA). Blasticidin, Normocin™, Zeocin®, CLI-095, and QUANTI-Blue™ Solution were purchased from Invivogen (San Diego, California, USA). All other chemicals were of analytical grade.

2.2 Cells

J774-Dual™ macrophage-like cell line was purchased from InvivoGen (San Diego, California, USA). The culture medium for the cultivation and splitting was DMEM which has a high D-glucose content. The medium was supplemented with 4 mM stable L-glutamine, 1 mM sodium pyruvate, 50 U/mL penicillin G, 50 µg/mL streptomycin, 100 µg/mL Normocin™ and 10% heat deactivated fetal bovine serum (FBS). As these cells are resistant to the selective antibiotics for eukaryotic and prokaryotic cells (Blasticidin and Zeocin®), they were added to the medium, every other week, to kill any possible cross-contamination. Cells were incubated in 5% CO2 and 95% humidified air at 37°C.

2.3 Methods

2.3.1 Preparation of NCs

To study the factors affecting the produced particle size, NCs were prepared by Wet ball milling (Chang, Zhan, Liang, & Liang, 2015), using a ball mill (Retsch GmbH, Haan, Germany). The Fluorescent dye (Lumogen® yellow S 0790) coarse powder was dispersed in aqueous solution of stabilizers, namely sodium dodecyl sulfate (SDS) and Hydroxypropylmethylcellulose (HPMC), to make a suspension with a concentration of 10

mg/ml. After that, the coarse suspension was added to the milling jar. Subsequently, zirconia (zirconium oxide) balls of different diameters and amounts were added to the jar and mixtures were milled for different time points at a frequency of 30 Hz.

Various process factors that affect the particle size of the NCs have been investigated. These include (a) the volume of stabilizer solution used to prepare the NC suspension, (b) drug to Zirconium beads weight ratio, (c) the diameter of the zirconium beads, (d) the SDS concentration in the stabilizer solution, (e) the HPMC concentration in the stabilizer solution, and (f) the milling time. Each factor was examined at 3 levels and for constant values of the other factors to determine the optimum level for each factor that yields the smallest particle size (Table 2-1). One of the SDS levels was selected to be lower than its critical micelle concentration (CMC), while the other 2 levels were above CMC.

Table 2-1. The factors affecting the particle size of the produced nanocrystals.

Factor	Values		
Volume of stabilizer solution (ml)	4	6	8
Drug to Zirconium beads weight ratio (mg/g)	1:1	1:1.5	1:2
Diameter of Zirconium beads (mm)	0.5	1	1.5
SDS concentration (%)	0.05	1.5	3
HPMC concentration (%)	1	2	3
Milling time (hr)	1	2	4

After optimization of the factors, anionic fluorescent, RX, and hemin NCs were prepared by dispersing the fluorescent dye (Lumogen® yellow S 0790), RX, or hemin coarse powder in aqueous solution of SDS (1.5% W/V) and HPMC (1% W/V), to make a dye or drug suspension with a concentration of 10 mg/ml. After that, the coarse suspension was added to the ball milling jar. Subsequently, zirconia balls, of a diameter of 1.5 mm, were added to the jar then the mixtures were milled at a frequency of 30 Hz to obtain nanosuspensions with a particle size of 100-500 nm.

RX-NCs of different charges were prepared by the same method using different surfactants and different time of milling to produce the desired particle size (Table 2-2). Anionic fluorescent and RX microcrystals (MCs) were prepared by reducing the time of milling time and the balls diameter to 2 min and 0.5 mm, respectively.

Table 2-2. The stabilizer solution and time of milling required to produce the tested formulations.

Type of formula	Stabilizer solution	Time
Fluorescent MCs	SDS 1.5%	2 min
	HPMC 1%	
Fluorescent NCs	SDS 1.5%	4 hr
	HPMC 1%	
Anionic RX-MCs	SDS 1.5%	2 min
	HPMC 1%	
Anionic RX-NCs	SDS 1.5%	6 hr
	HPMC 1%	
Cationic RX-NCs	CTAB 0.5%	12 hr
	HPMC 1%	
Non-ionic RX-NCs	P188 6%	8 hr
	HPMC 1%	
Hemin-NCs	SDS 1.5%	4 hr
	HPMC 1%	

2.3.2 Characterization of the prepared NCs and MCs

Average particle diameter (Z-average) and the polydispersity index (PDI) of the prepared formulations were assayed by dynamic light scattering (DLS) at 173° angel, and Zeta potential was measured by laser Doppler electrophoresis (LDE) using (Nanopartica SZ-100, Horiba, Kyoto, Japan). All samples were measured in triplicates and results are expressed as the average ± standard deviation.

Morphological evaluation of the nanocrystals was done using a scanning electron microscope (SEM) (Helios G4 CX, Thermo Fisher Scientific, USA) after being washed with distilled water, diluted, and air dried. The dried samples were affixed and assembled onto sample plates and then sputter coated with gold under vacuum conditions.

2.3.3 Multiple linear regression of the factors affecting the particle size

The Pearson correlation coefficient between the factors studied was determined using GraphPad Prism software to determine if multicollinearity existed between the factors studied that could affect the regression model to be used subsequently. A multiple linear regression was then determined using GraphPad Prism software and the regression equation, actual versus predicted particle size, and residuals plot were developed.

2.3.4 In vitro cytotoxicity of RX and hemin formulations

J774-DUAL™ cells were seeded in 96-well plates at 10^5 cell/well and left to adhere for 24 hr. After that, the medium was changed with lipopolysaccharide (LPS) solution (100 ng/ml) in the culture medium to activate the macrophage cells. On the third day, different concentrations of RX-NCs, RX solution (in PEG 400 : Water : Glycerin 86:9:5), hemin solution (in DMSO) or hemin NCs were added to the cells and then incubated for another 24 hr. MTT assay was performed, on the fourth day, to determine the cell survival, and the LC_{50} values were assessed using GraphPad prism software.

2.3.5 In vitro evaluation of RX formulations' efficacy

J774-DUAL™ cells can produce a secreted embryonic alkaline phosphatase (SEAP) into the supernatant of cell culture when activated with pathogen-associated molecular patterns (PAMPs) e.g., LPS under the control of the transcription factor Nuclear Factor-κB (NF-κB). SEAP can be measured colorimetrically after adding QUANTI-Blue™ Solution. Therefore, cells were seeded at 10^5 cell/well in 96-well plates and left to adhere for 24 hr. consequently, the old media was replaced by LPS solution (100 ng/ml) in culture medium, to activate the cells to produce SEAP, and plates were then incubated for another 24 hr. On the third day, cells were treated with different concentrations of RX solution (in PEG 400 : Water : Glycerin 86:9:5), RX-MCs, or RX-NCs for 24 hr. The concentrations of RX were selected after the determination of the cytotoxicity for all the different formulations using MTT assay. Some wells were treated

with drug-free medium to serve as negative controls. finally, on the fourth day, QUANTI-Blue™ solution was added to the cells' supernatant in another plates, incubated for 6 hours, then measured at 650 nm using a plate reader (PerkinElmer, Massachusetts, USA). To normalize the SEAP results to each well's protein content, Lysis buffer was added to the cells and the protein content was determined with Micro BCA™ Protein Assay Kit (Thermo-Fischer scientific) according to manufacturer's instructions. The results were expressed as a percent inhibition of SEAP production compared to the untreated control. This was obtained by dividing the difference, between the normalized absorbance of the untreated cells and that obtained from treated cells, by the normalized absorbance of untreated cells.

2.3.6 In vitro Anti-inflammatory effect of hemin formulations

J774-DUAL™ cells were seeded in 96-well plates at 10^5 cell/well and left to adhere for 24 hr. After that, the medium was changed with LPS solution (100 ng/ml) in the culture medium, to activate the cells to produce SEAP, and incubated for another 24 hr. On the third day, cells were treated with either hemin solution in DMSO or hemin NCs diluted with culture medium at different hemin concentrations then incubated for 4,12, and 24 hours. Finally, the percent inhibition of SEAP was determined as previously mentioned.

To determine the effect of hemin NCs and solution on inactivated cells, the attached cells were not activated with LPS on the second day, instead fresh medium was added and left for the following day, then the cells were incubated with the hemin formulations for 24 hours, then SEAP amount was assessed as before.

A third experiment was performed to determine if the difference in effect between NCs and solution was related to Toll-like receptor 4 (TLR4) activation. In this experiment, after activation of cells with LPS, cells were incubated with CLI-09 (TLR4 inhibitor) for 12 hours before administration of hemin NCs and solution. After treatment, the cells were incubated for 24 hours. The results of these two experiments were expressed as the normalized amount of SEAP produced by the cells, which may indicate the degree of NF-κB activation.

2.3.7 Bioadhesion of MCs and NCs to the Inflamed Colonic tissue

Experiments were performed at the University of Burgundy/Franche-Comté in Besançon, France, under experimental permit No. A-25-48 in accordance with French legislation on animal experimentation. Bioadhesion test was done as previously reported (Lamprecht et al., 2001). Briefly, experimental colitis was induced in swiss albino male mice of four weeks old

(average weight: 30 g) by the intrarectal injection of 100 ml of Trinitrobenzenesulfonic acid (TNBS) in 50% ethanol (90 mg/kg body weight, 3 cm from the anus). Mice were divided, 1 day after colitis induction, into 2 groups (n=5). One group received intrarectally a 0.1-ml MCs suspension of Lumogen® yellow (12.5 mg particles/kg body weight) for three days, while the other group received equal volume and doses of NCs suspension. After that, a washout period was done, during which no formulation was administered, to allow unadhered NCs to be washed out from the colon. Therefore, mice were sacrificed only 72 hours after the last dose and colons were resected.

After resection of the colon, longitudinal cuts were made and rinsed carefully to get rid of food residues. The inflamed areas which have macroscopic damages of the mucosal tissue, were separated from the noninflamed tissue before colon segmentation and lyophilization in the dark to avoid bleaching of the fluorescent dye. Subsequently, dried tissue was incubated in a shaking water bath with 10 ml of chloroform at 30°C in the dark for 24 h. Then to ensure complete extraction of the dye, the extraction procedure was performed two times. The dye solutions thus produced were diluted to a total volume of 100 ml with chloroform and then analyzed by fluorescence spectroscopy.

2.3.8 Evaluation of RX formulations' efficacy in murine colitis

Swiss albino mice (male, average weight: 30 g, and of age: 4 Weeks) were divided into 11 groups, ten colitis groups, and one healthy group (n=6). Experimental colitis was induced as mentioned before. After 1 day of colitis induction, one group received isotonic saline solution and served as colitis control group, while the other mice were divided according to treatment type and the daily dose they rectally received into: (a) RX solution at 5mg/Kg, (b) Anionic RX-MCs at 1mg/Kg, (c) Anionic RX-MCs at 2mg/Kg, (d) Anionic RX-MCs at 5mg/Kg, (e) Anionic RX-NCs at 1mg/Kg, (f) Anionic RX-NCs at 2mg/Kg, (g) Anionic RX-NCs at 5mg/Kg, (h) Cationic RX-NCs at 5mg/Kg, and (i) Non-ionic RX-NCs at 5mg/Kg. Healthy mice received saline solution. All treatments were administered rectally for 3 days. 24 hours after the last treatment, all mice were sacrificed then colons were resected. After rinsing the colons, they were measured (weight and length), then minced, and used for further biochemical analyses.

The anti-inflammatory effect was evaluated on the basis of (1) Clinical activity score (CAS), which includes animal body weight loss, stool consistency, and rectal bleeding during the treatment period, (2) The ratio of colon wet mass (g) to colon length (cm), (3)

Myeloperoxidase (MPO) activity (unit) per wet tissue weight (g), and (4) Cytokine levels, including interleukin-1β (IL-1β) and tumor necrosis-α (TNF-α), which were determined using ELISA in tissue homogenates according to the manufacturer's protocols.

2.3.9 Factor Xa blood levels

To determine whether the administration of RX solution, NCs, or MCs would result in lowering of Factor Xa in blood, systemic Factor Xa levels were determined in plasma samples, taken 15 hours after rectal administration to ensure complete absorption and effect, using the Mouse Coagulation Factor X Total Antigen assay Elisa Kit (Molecular Innovations, Peary Court, Novi, MI, USA).

2.3.10 Anti-inflammatory effect of hemin in experimental colitis

To determine anti-inflammatory effect of hemin formulations, the Swiss albino mice (male, 4 Weeks, and average weight, 30 g) were divided into 7 groups, one healthy group and six colitis groups (n=6). The six colitis groups were divided, according to the dose, site of injection, and the formulation type, into (a) Intraperitoneal (IP) injection of hemin solution at 10 mg /Kg, (b) Intrarectal injection of hemin solution (in a buffer of pH 8) at 5 mg /Kg, (c) Intrarectal injection of hemin Solution at 10 mg /Kg, (d) Intrarectal injection of hemin NCs at 5 mg /Kg, (e) Intrarectal injection of hemin NCs at 10 mg /Kg, and (f) Colitis control group which received isotonic saline solution. All treatments were started 24 days after colitis induction and for 3 days, then the mice were sacrificed 24 hours after the last treatment and the colons were resected, rinsed, measured (weight and length), minced, and finally underwent biochemical analyses. The anti-inflammatory effect was assessed as described previously with RX experiment (2.3.8)

2.3.11 Statistical analysis

During the optimization of the factors affecting the particle size of the NCs, one-way analysis of variance (ANOVA) followed by Tukey's comparison test was performed using GraphPad Prism software to determine the statistical significance of the differences between the particle sizes of the prepared formulations. Pearson correlation and multiple linear regression were applied using GraphPad Prism software to model the relationship between the factors and predict the particle sizes used for the three-dimensional surface plots.

To determine the significance of differences between all groups of in vivo and in vitro experiments, one-way analysis of variance (ANOVA) followed by Tukey's comparison test was performed using GraphPad Prism software. All results are expressed as mean ± SD of triplicate experiments.

3 Study of the factors affecting NCs' particle size

3.1 Results

3.1.1 Optimization of the factors affecting particle size

3.1.1.1 Volume of stabilizer solution

At constant values of the other factors studied, changing the volume of the stabilizer solution (4, 6, and 8 ml) showed no effect on the particle size of the NCs produced (Figure 3-1). The mean particle sizes produced were in the range of 268.5 to 284.5 nm. due to insignificant difference 4 ml of stabilizer solution was selected for the further studies of the other factors.

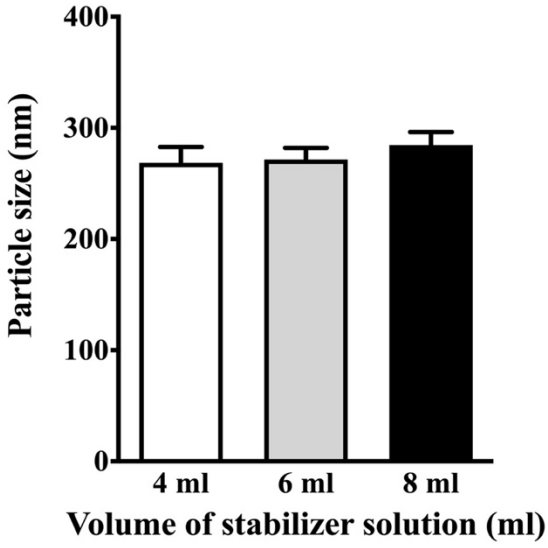

Figure 3-1. The effect of the volume of the stabilizer solution on the particle size of NCs.

3.1.1.2 Drug to beads weight ratio

Different weights of zirconium beads were added to the milling jar to achieve drug to bead weight ratios (mg/g) of 1:1, 1:1.5, and 1:2, while other factors were kept at constant levels. It was found that the drug to bead weight ratio had no effect on the particle size of the NCs produced. The average particle sizes ranged from 230.7 to 286.5 nm (Figure 3-2). Owing to the insignificant difference in particle size and to ensure adequate filling of the milling jar, a weight ratio of 1:2 between drug and beads was selected for further studies.

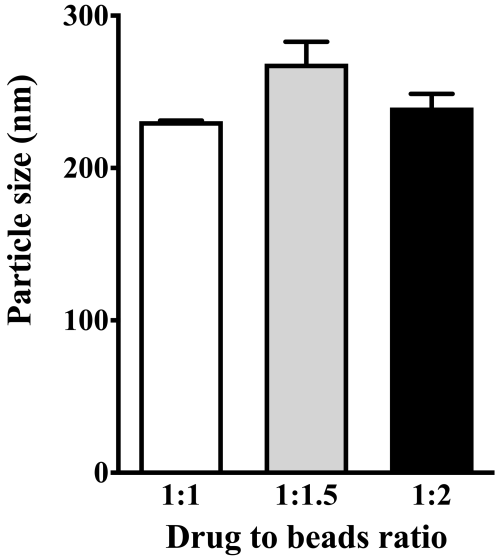

Figure 3-2. The effect of drug to beads weight ratio on the particle size of NCs.

3.1.1.3 diameter of the beads

Zirconium beads of different diameter were studied (0.5, 1, and 1.5 mm) , where it was found that by increasing the diameter of the used beads, NCs of smaller particle size is produced (Figure 3-3). Therefore, beads of 1.5 mm diameter were selected for the further study of the other factor.

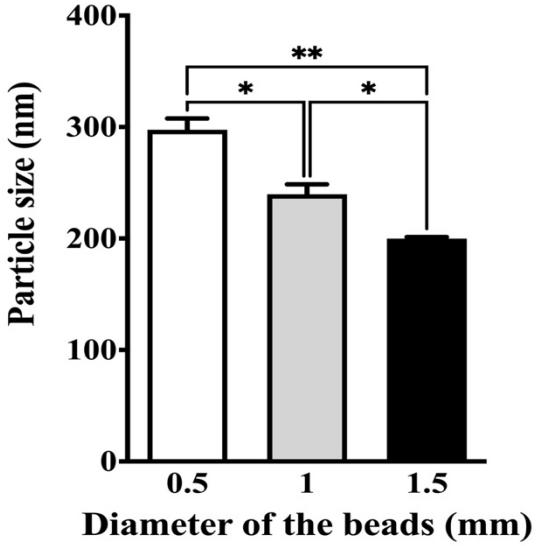

Figure 3-3. The effect of the beads' diameter on the particle size of NCs.
*** and ** indicate statistically significant differences. (* for p<0.05 and ** for p<0.01).**

3.1.1.4 SDS concentration in stabilizer solution

One SDS concentration was chosen to be below the CMC (0.2%), while the other two concentrations were above the CMC to test the effects of micelle formation on the particle size of the NCs formed. It was found that at SDS concentration below the CMC (0.05%), the NCs produced were larger than at concentrations above the CMC (1.5 and 3%). Moreover, there was no significant difference between the two concentrations above the CMC (Figure 3-4). Therefore, the lowest of these two SDS concentration (1.5%) was selected for further investigations.

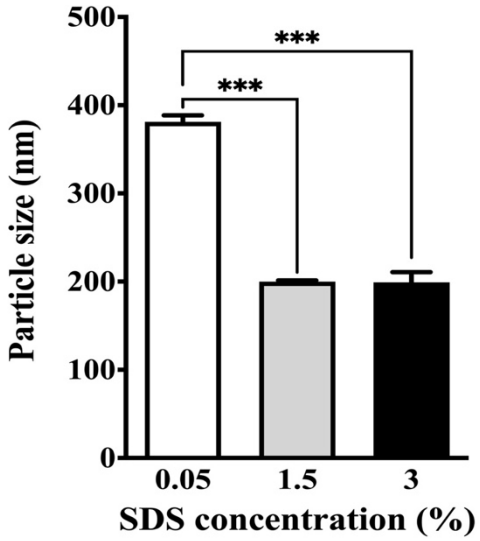

Figure 3-4. The effect of SDS concentration in stabilizer solution on the particle size of NCs. * indicates a statistically significant difference ($p<0.001$).**

3.1.1.5 HPMC concentration in stabilizer solution

After testing different HPMC concentrations (1, 2, and 3%), it was found that the particle size of the prepared NCs increased with increasing HPMC concentration (Figure 3-5). Therefore, the lowest concentration of HPMC (1%) was chosen for further factor evaluation.

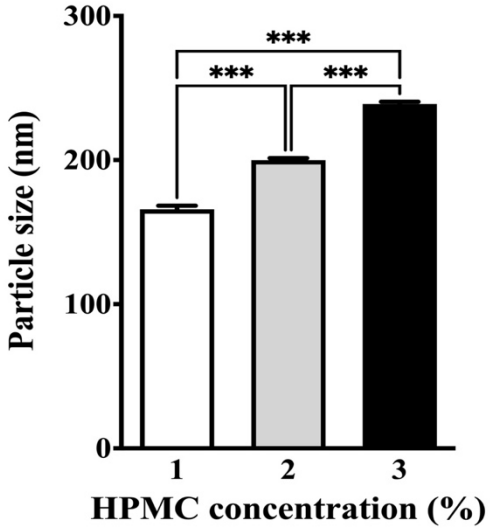

Figure 3-5. The effect of HPMC concentration in stabilizer solution on the particle size of NCs. * indicates a statistically significant difference (p<0.001).**

3.1.1.6 Milling time

The effect of the milling time was assessed at three levels (1, 2, and 4 hours). The particle size of the prepared NCs was inversely related to the time of milling (Figure 3-6). Therefore, longer milling times were used to produce smaller NCs of drugs in the following studies.

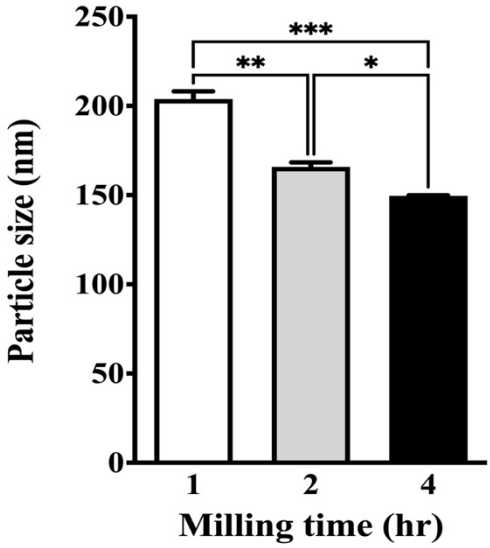

Figure 3-6. The effect of milling time on the particle size of NCs.
***, **, and *** indicate statistically significant differences. (* for p<0.05, ** for p<0.01, and *** for p<0.001).**

3.1.2 Multilinear regression of the factors affecting the particle size

The Pearson correlation determination indicated no strong correlation between any of the studied factors (Figure 3-7).

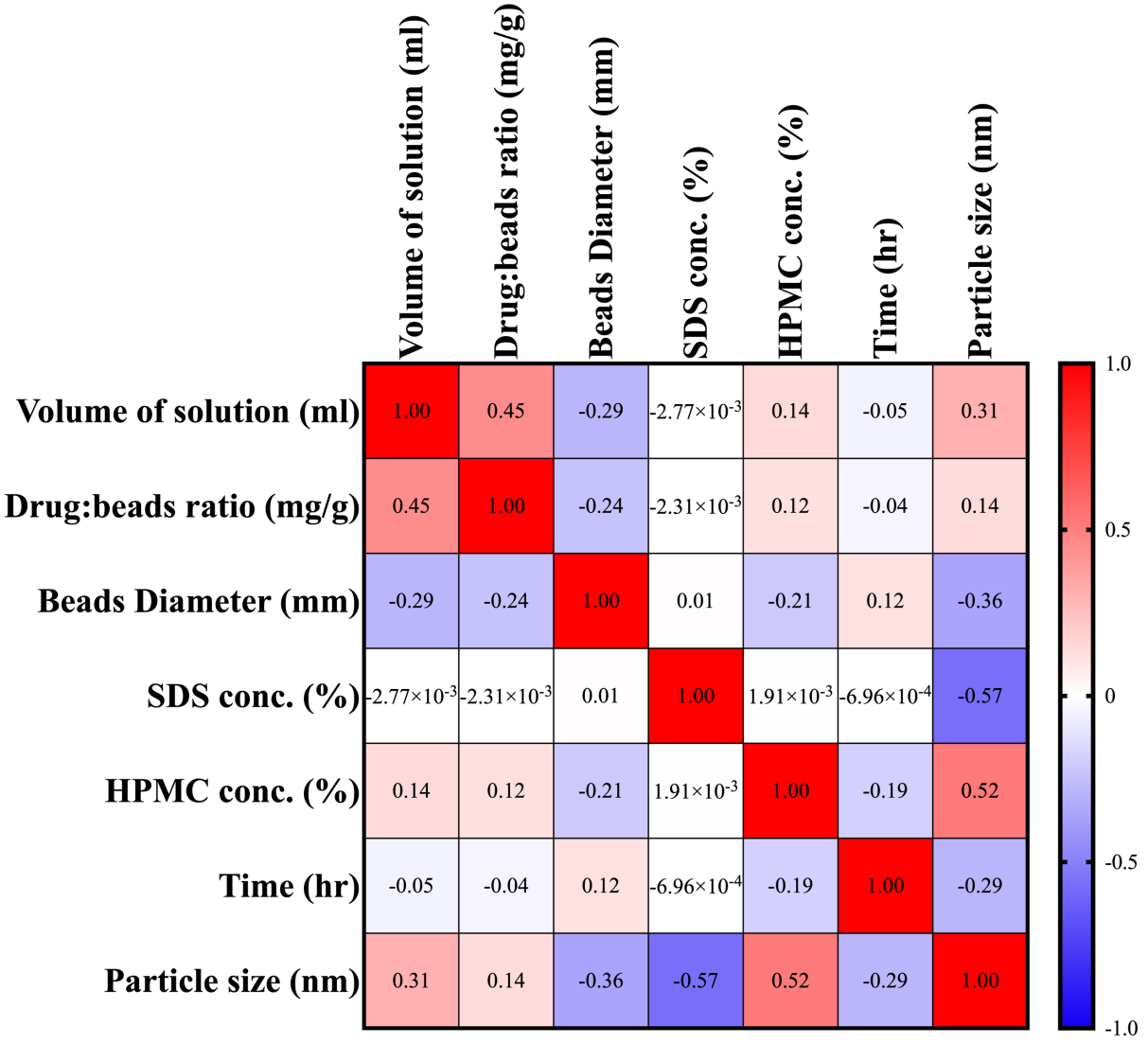

Figure 3-7. The Pearson correlation matrix between the studied factors affecting the particle size of NCs.

The absence of multicollinearity between the factors allowed for the performance of the multiple linear regression. The developed linear equation was as follows:

Particle size (nm) = 283.6 + 10.42·A – 4.499·B – 37.52·C – 61.01·D + 46.49·E – 18.06·F

Where:

A: Volume of solution (ml)

B: Drug : beads ratio (mg/g)

C: Beads Diameter (mm)

D: SDS conc. (%)

E: HPMC conc. (%)

F: Time of milling (hr)

The actual vs predicted particle size graph was developed (Figure 3-8) and indicated a good relation between them as indicated by the distribution of residuals around the zero line in the residual plot (Figure 3-9). In addition, the residuals passed Kolmogorov-Smirnov (distance) normality test.

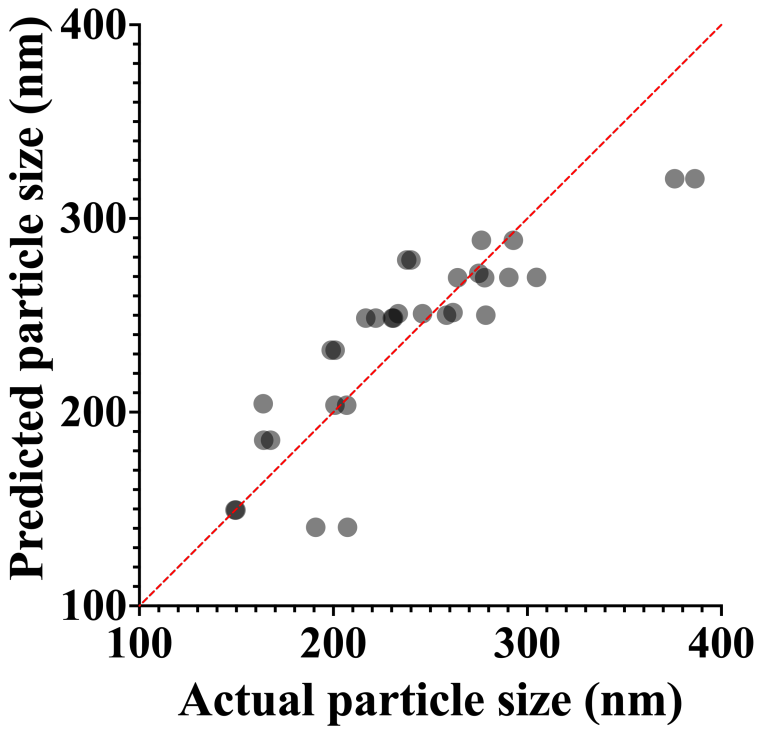

Figure 3-8. The actual vs predicted values of particle size after multilinear regression

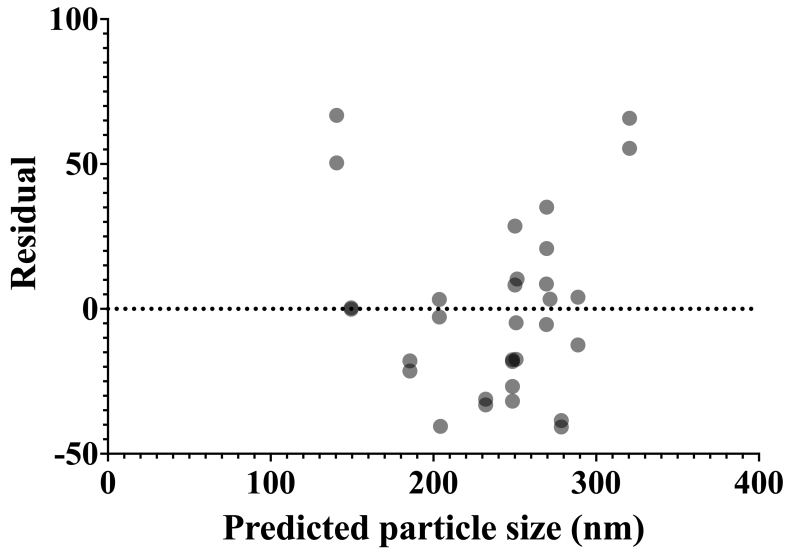

Figure 3-9. The residual plot of predicted particle size after multilinear regression.

From the regression model, three-dimensional surface plots were set up between the most significant factors, namely SDS concentration, HPMC concentration, Beads diameter, and Time of milling (Figure 3-10 to 3-15).

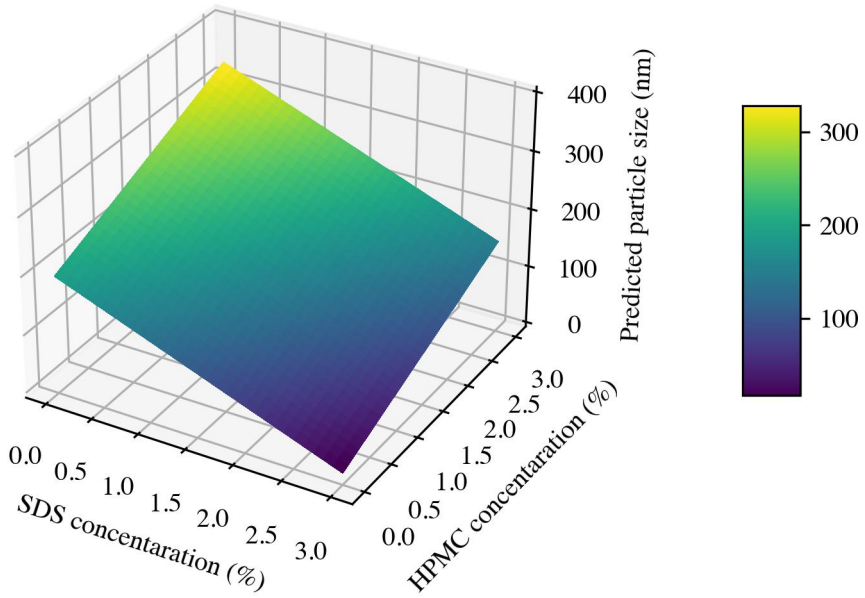

Figure 3-10. Three-dimensional surface plot showing the effect of HPMC concentration and SDS concentration on predicted particle size.

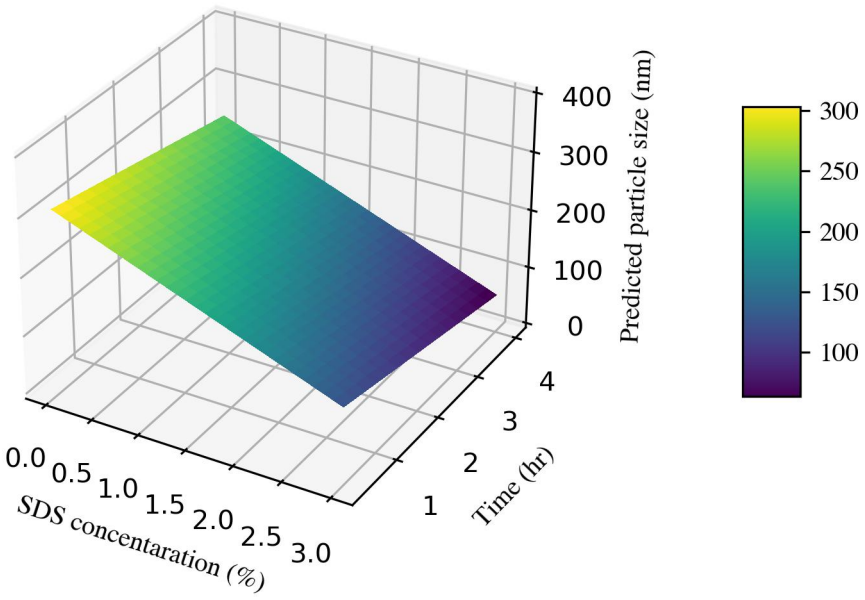

Figure 3-11. Three-dimensional surface plot showing the effect of SDS concentration and time of milling on predicted particle size.

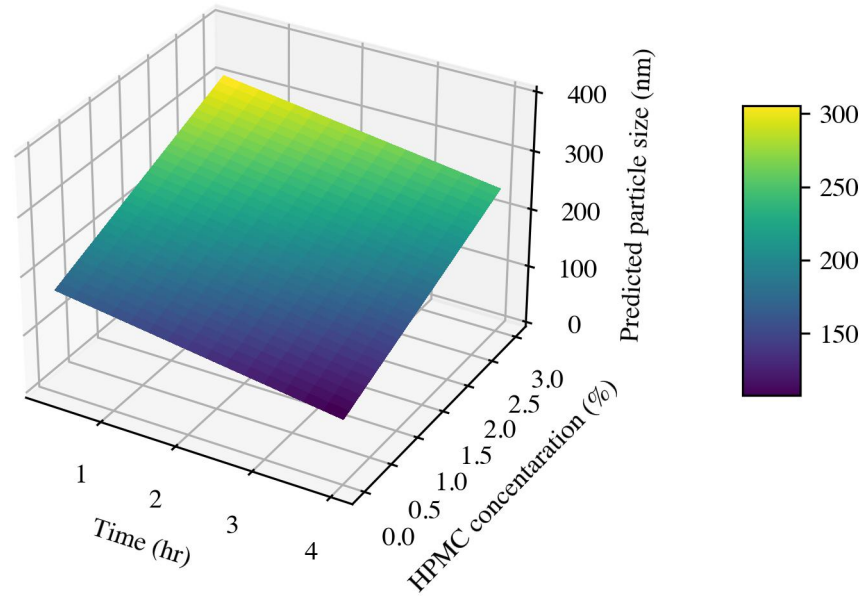

Figure 3-12. Three-dimensional surface plot showing the effect of time of milling and HPMC concentration on predicted particle size.

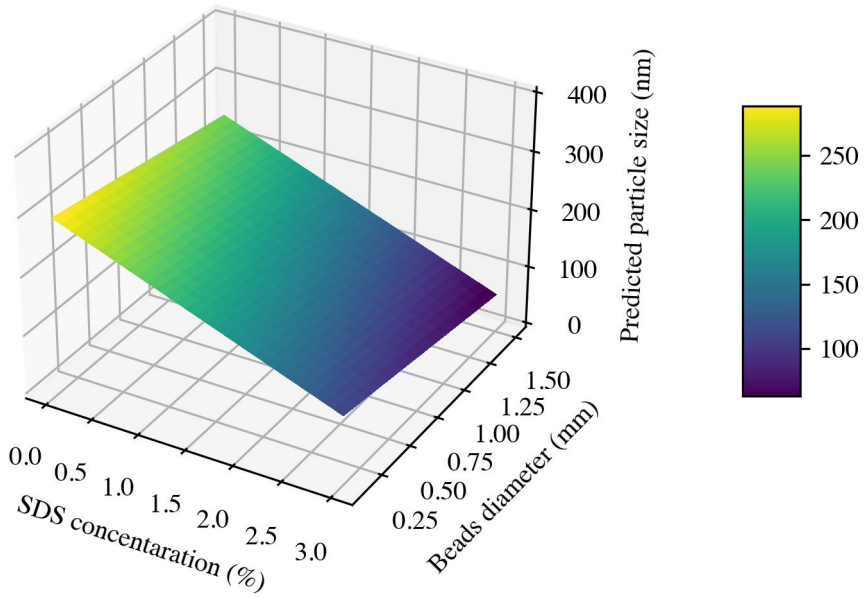

Figure 3-13. Three-dimensional surface plot showing the effect of SDS concentration and beads diameter on predicted particle size.

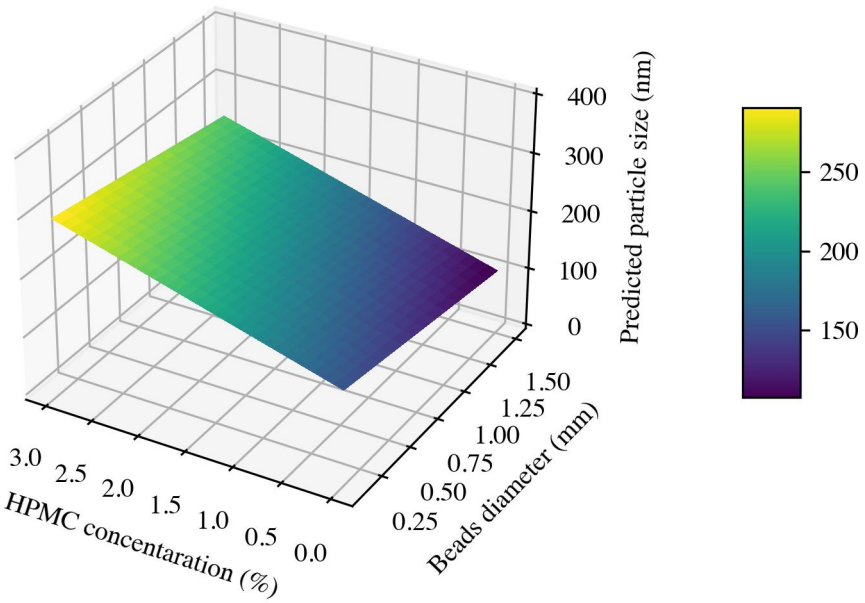

Figure 3-14. Three-dimensional surface plot showing the effect of HPMC concentration and beads diameter on predicted particle size.

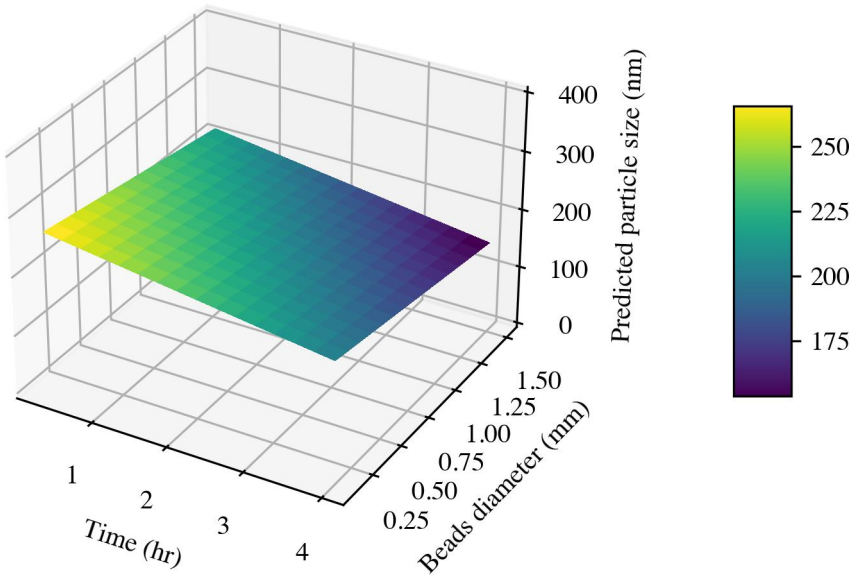

Figure 3-15. Three-dimensional surface plot showing the effect of time of milling and beads diameter on predicted particle size.

3.2 Discussion

NCs suspensions are an unstable system thermodynamically due to their high interfacial area and therefore have a high free energy of the interface. Therefore, The function of the stabilizer solution in the formation of nanocrystals is to stabilize the surface of the individual nanocrystals to prevent them from agglomerating into larger particles (Kim & Lee, 2010). The stabilization mechanisms of stabilizers include electrostatic repulsion and steric effect stabilization. Therefore, the selection of a charged stabilizer solution could be of great value in this context, as the repulsion between particles covered with the similar charge will increase the stability of the NCs suspension (Narayan et al., 2017). Yet, the choice of the concentration of the charged stabilizer is challenging, as a low concentration may not have a stabilizing effect and a high concentration is not necessary and could reduce the safety of these formulations. In our study, SDS at a concentration above the CMC resulted in a higher stabilization effect than at a lower concentration. However, further increasing the SDS concentration had no more effect, which may indicate that the SDS concentration of 1.5% resulted in complete coverage and stabilization of the NCs.

Reducing the volume of the stabilizer solution could increase the impact force by allowing a larger headspace in which the grinding media can move freely. However, a larger headspace also means more air and more opportunities for formation of foam in the grinding vessel due to agitation, which does not allow for much attrition. This could be the explanation for the fact that the different amount of surfactant has no influence on the particle size produced.

In some studies, there is a direct relationship between the diameter of the milling beads and the particle size obtained (Date et al., 2018). However, in our and other studies (Narayan et al., 2017), there was an indirect relationship between them. This could be due to the two forces that cause the milling effect. While an increase in diameter of the milling beads means an increase in the weight of each bead, which in turn increases the impact force of each bead, a decrease in diameter can translate into a larger number of beads and increases the number of impacts per movement. therefore, the net result of these forces may vary among studies.

To increase the stability of the NCs suspension, steric stabilization with HPMC was also performed. The effect of HPMC concentration on the particle size produced was as expected, since increasing the HPMC concentration increases the viscosity of the stabilizer solution, which forms a resistance to the movement of the beads and thus reduces their impaction and attrition effects on the coarse drug particles resulting ultimately in larger NCs.

The milling time was important in our study as well, since with increasing time, a greater number of impacts and crushing occurs in the ball mill. As reported before, the size of the produced NCs decreases with increasing time until it reaches an optimum size after which the particle size of the produced NCs no longer decreases (Date et al., 2018). This is due to the initial crystal breakage due to cracks and crystal defects in larger crystals, which can be relatively easily broken into smaller crystals. Thereafter, crushing continues at a slower rate until all defects and cracks are broken. in our study it was feasible to reach the optimum size before reaching the time limit. Therefore, there was no need for more time of milling.

The multiple linear regression performed shows the contribution of each factor to the particle size produced. The signs in the linear equation suggest that HPMC concentration (%) and stabilizer solution volume are directly related to particle size (positive sign in the linear equation). However, all other factors are inversely related to particle size. The value of the coefficients in the equation indicates that the order of the effect produced by the various factors on the particle size was as follows: SDS concentration (%) > HPMC concentration (%) > bead diameter (mm) > time (hr) > volume of solution (ml) > drug : bead ratio (mg/g). This can also be deduced from the slope of the plot of the three-dimensional surface plots of the four most important factors. It can be observed that the slope of the plot in the case of HPMC with SDS concentration is higher than all other curves since they have the greatest influence on the particle size. This indicates the good fit of the model and its ability to predict the particle size from the factors. The determination of the most important factors facilitated the use of their optimum values for the preparation of drug NCs.

4 Rivaroxaban nanocrystals

4.1 Results

4.1.1 Preparation and characterization of NCs

Fluorescent and RX-NCs of different surface charge were prepared and characterized for particle size and zeta potential. The particle size of RX-NCs were in the range between 200-500 nm and the zeta potential measurement confirmed the intended preparation of differently charged NCs (Table 4-1). SEM images showed that RX-NCs of different surface charge had irregular shape and almost the same size distribution. Fluorescent NCs showed comparable shape and size distribution to RX-NCs (Figure 4-1).

Table 4-1. The particle sizes, PDI, and zeta potential of the tested formulations.

Type of formula	Particle size (nm)	PDI	Zeta Potential (mv)
Fluorescent MCs	1058.9 ± 167.9	0.498 ± 0.060	-29.9 ± 0.5
Fluorescent NCs	149.5 ± 2.4	0.306 ± 0.048	-27.9 ± 8.4
Anionic RX-MCs	6819.0 ± 1093.6	1.447 ± 0.173	-20.9 ± 5.3
Anionic RX-NCs	215.1 ± 8.1	0.302 ± 0.019	-23.7 ± 1.3
Cationic RX-NCs	241.3 ± 18.8	0.263 ± 0.051	22.1 ± 3.8
Non-ionic RX-NCs	459.8 ± 21.3	0.202 ± 0.087	0.5 ± 0.5

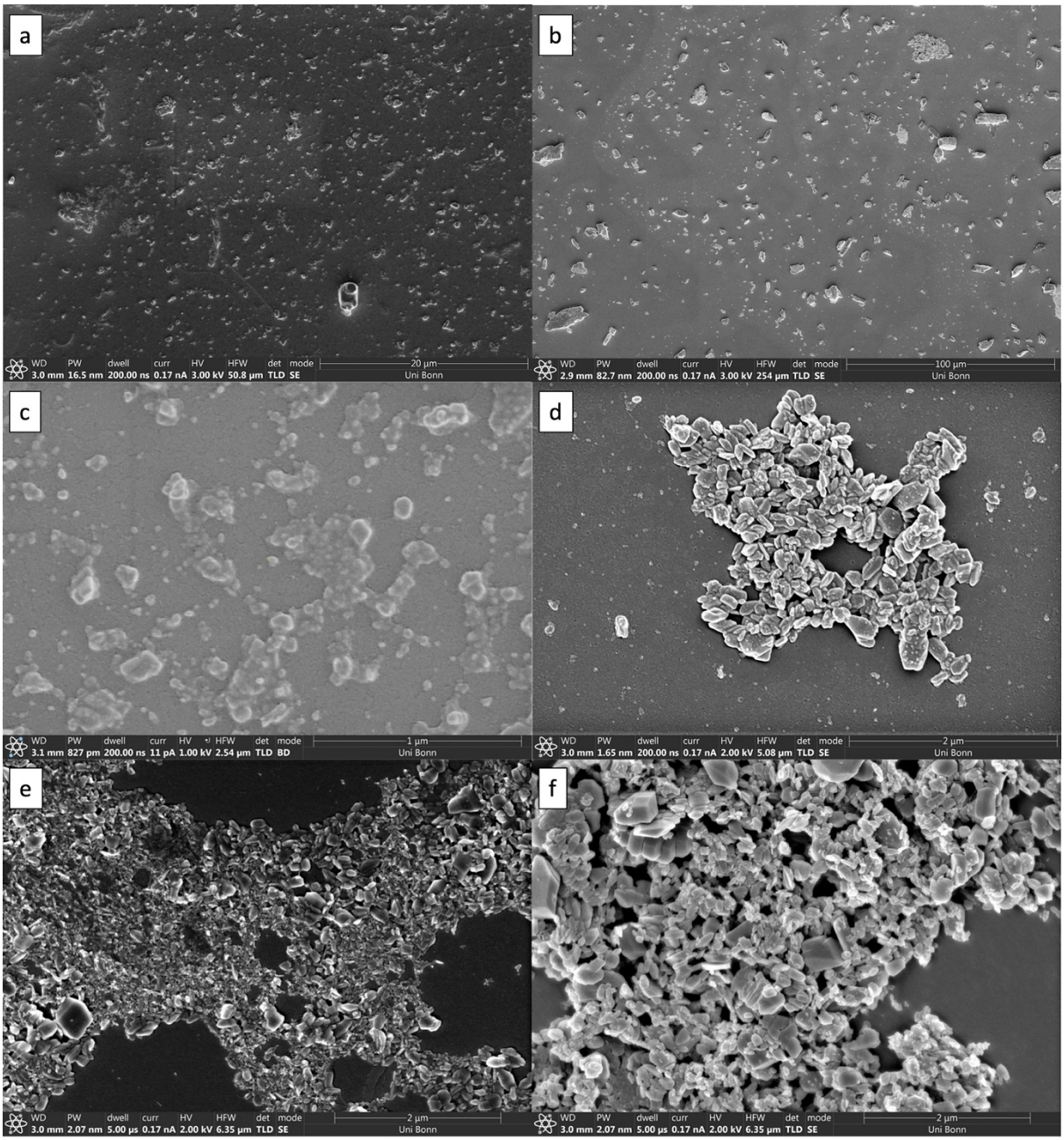

Figure 4-1. SEM images of (a) fluorescent MCs, (b) anionic RX-MCs, (c) fluorescent NCs, (d) anionic RX-NCs, (e) cationic RX-NCs, and (f) nonionic RX-NCs.

4.1.2 In vitro evaluation of RX formulations' efficacy

After MTT testing all RX formulations on J774-DUAL™ cells, RX concentrations that resulted in 70% or greater viability were selected for each formulation (Figure 4-2). These safe concentrations were then used to determine anti-inflammatory activity.

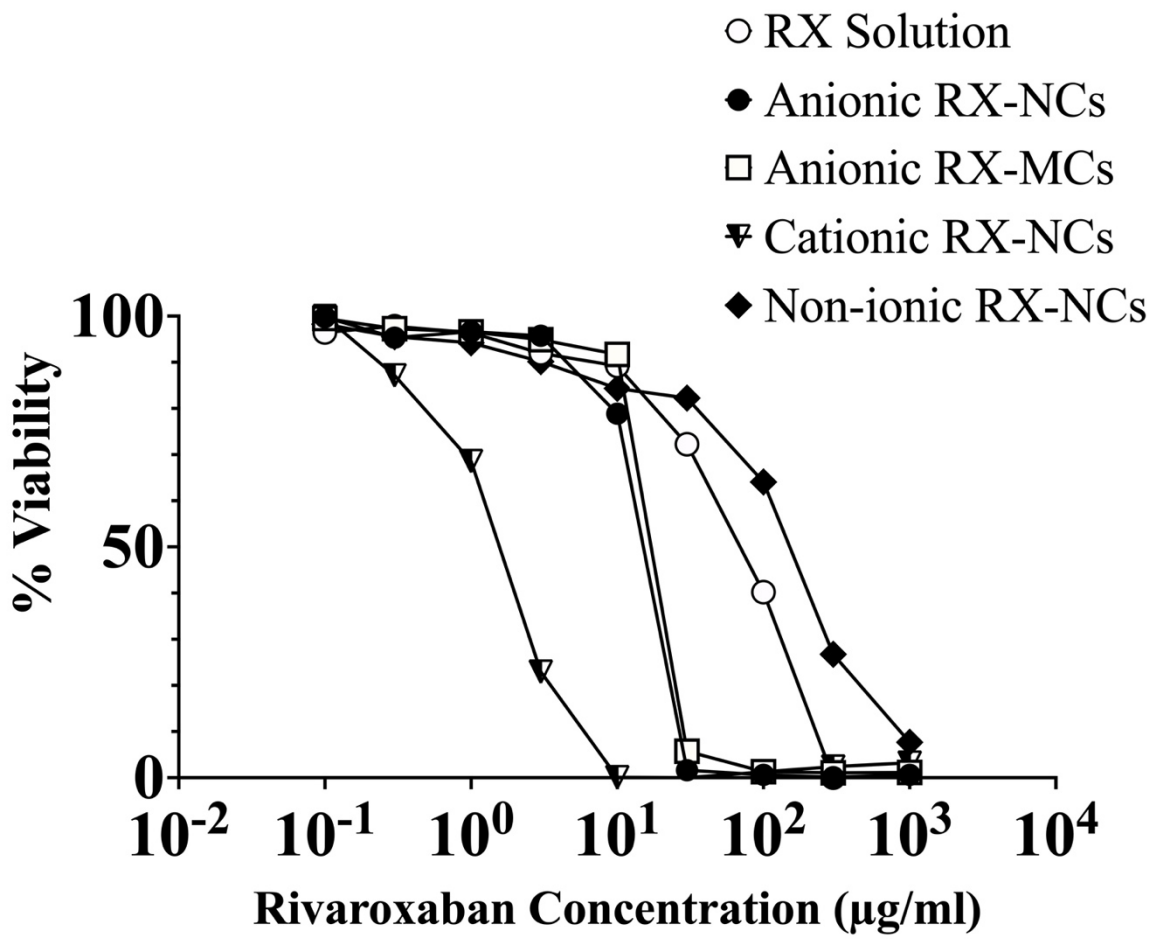

Figure 4-2. Cell viability determined by MTT assay after application of RX formulations for 24 hr.

Only anionic NCs and MCs were able to produce enhanced anti-inflammatory effects in terms of inhibition of SEAP production (about 54%). It was also found that there was no difference between them in the anti-inflammatory activity (Figure 4-3).

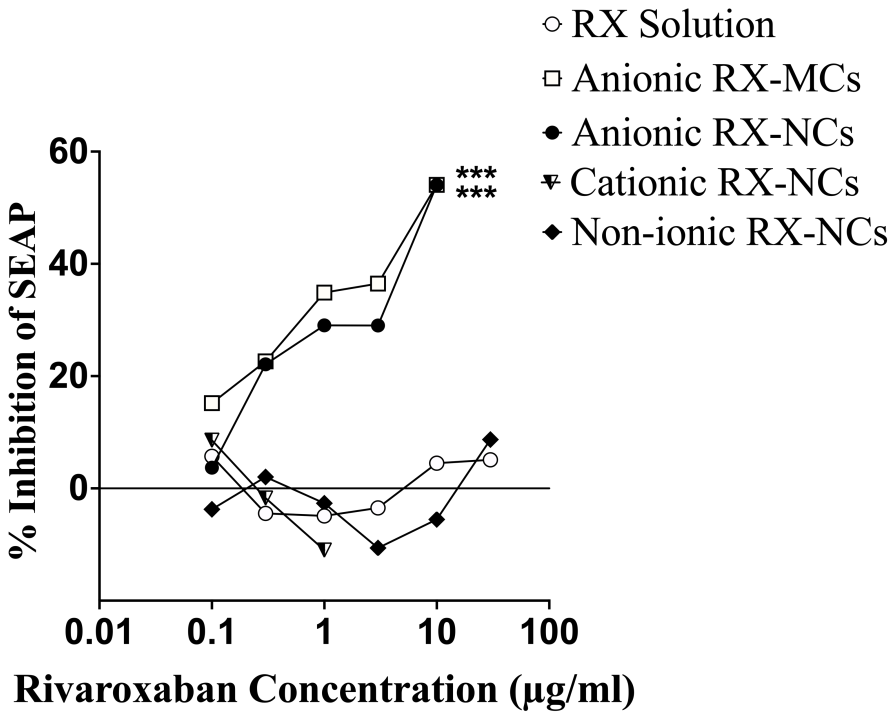

Figure 4-3. The anti-inflammatory effect of RX nanocrystals, microcrystals, and solution on J774-DUAL™ cells after activation with LPS (error bars removed for clarity).

***** indicates a statistically significant difference compared to the RX solution ($p<0.001$).**

4.1.3 Bioadhesion of NCs and MCs to Inflamed Colonic tissue

The percentage of adhering fluorescent NCs and MCs to inflamed and non-inflamed tissue was assessed. In both cases, the percentage of NCs or MCs adhering to inflamed tissue was higher than that to non-inflamed tissue (Figure 4-4). NCs and MCs showed almost 15- and 13-fold higher percentages of particle adhesion to inflamed tissue than to healthy tissue, respectively. At the same time, there was a significant difference between NCs and MCs in terms of the percentage of adherent formulations to inflamed tissue. CLSM images showed that NCs had a broader fluorescence distribution within the colon tissue than MCs, which were mainly located in the lumen of the colon (Figure 4-5).

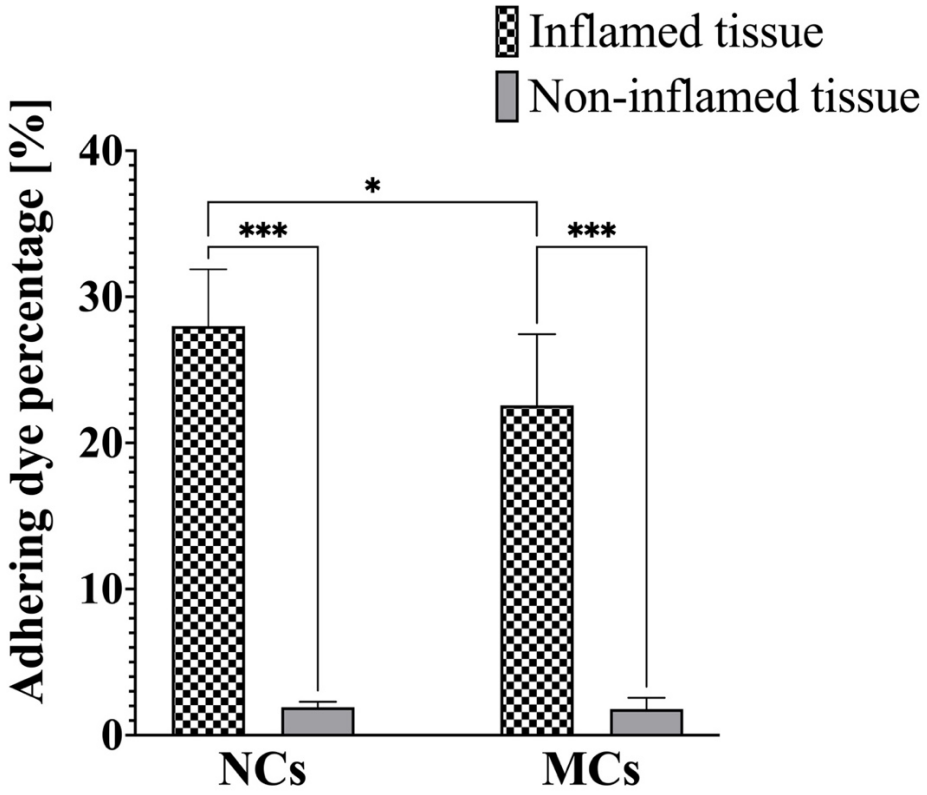

Figure 4-4. The percentage of adhering fluorescent NCs and MCs to inflamed and non-inflamed colon tissue.

*** and *** indicate a statistically significant difference (* for $p<0.05$ and *** for $p<0.001$).**

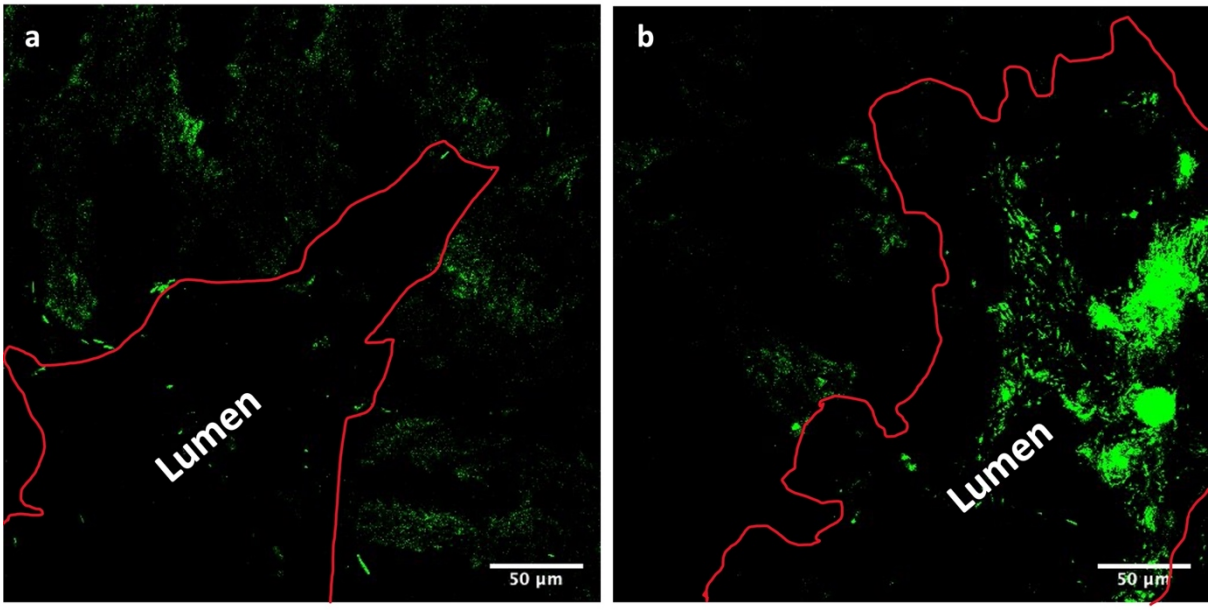

Figure 4-5. The CLSM imaging of fluorescent (a) NCs and (b) MCs in inflamed colon tissue. Red lines are the lumen's borders.

4.1.4 Evaluation of RX formulations' efficacy in murine colitis

After evaluation of CAS values, it was found that CAS values for anionic RX-NCs and RX-MCs groups were lower than in the colitis control group (Figure 4-6). The significance level of difference from colitis control was lower for solution and all doses of MCs than for the corresponding doses of NCs.

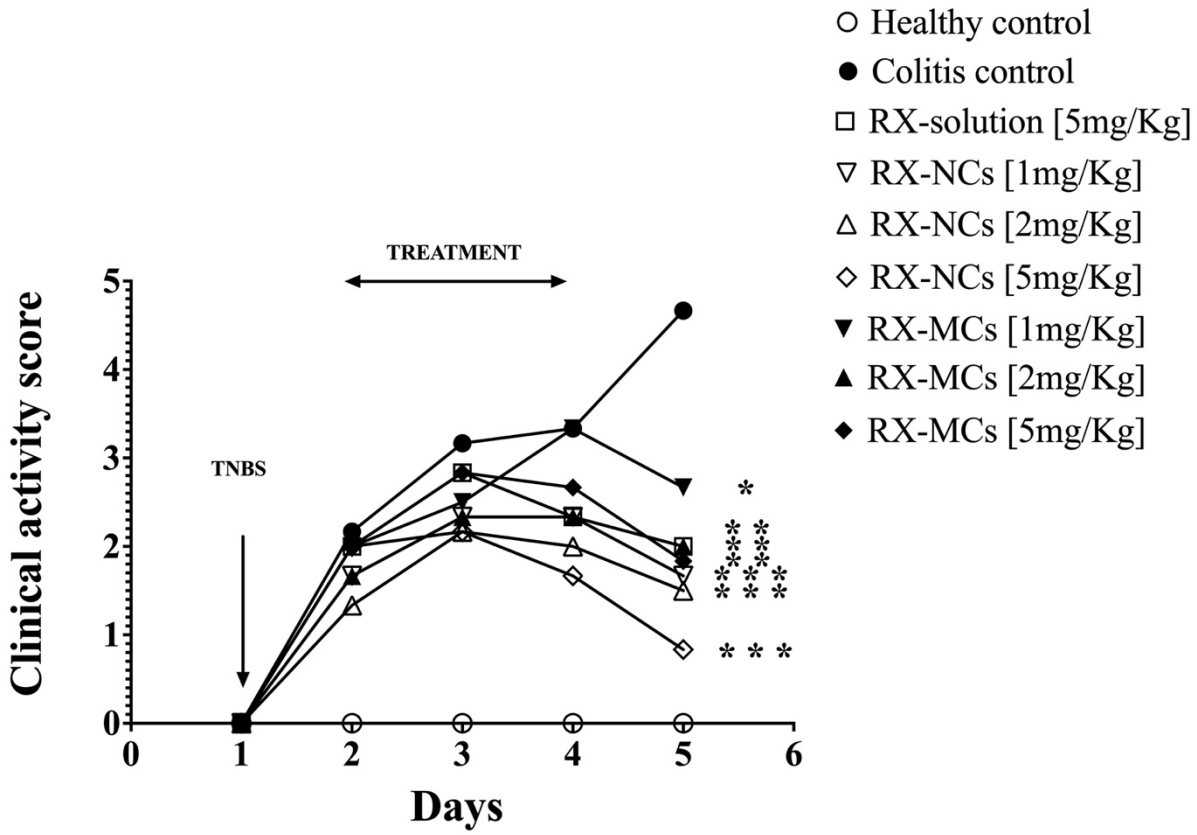

Figure 4-6. Clinical activity score of RX solution, NCs, and MCs treated groups at different doses (Error bars removed for clarity).

***, * * and * * * indicate a statistically significant difference compared to colitis control group (* for p<0.05, ** for p<0.01, and *** for p<0.001)**

Moreover, the weight to length ratio was significantly smaller for all treated groups than the colitis control group. However, there was no significant difference between the groups treated with different formulations i.e., solution, anionic NCs and MCs, and at different dose levels (Figure 4-7 a).

MPO levels were lower in all treated groups, except RX solution, than in the colitis control group. Moreover, anionic RX-NCs and RX-MCs at all doses were able to significantly lower the MPO levels than RX-solution at the highest dose (5 mg/Kg). However, there was also

no significant difference between groups treated with either different doses or different crystal size, i.e., MCs or NCs (Figure 4-7b).

Anionic RX-NCs at a dose of 5 mg/Kg was the only group that showed significantly lower TNF-α levels compared with the colitis control group (Figure 4-7c). However, all treated groups, with different doses, showed significantly decreased IL-1β levels compared with the colitis control group (Figure 4-7d). In both cases, there was no difference between groups treated with anionic NCs or MCs, at all doses, and the group treated with solution. Due to its significant effect in reducing the MPO and TNF-α levels, RX-NCs at a dose of 5 mg/kg were selected for further evaluation of the effects of surface charge on their activity in the treatment of colitis.

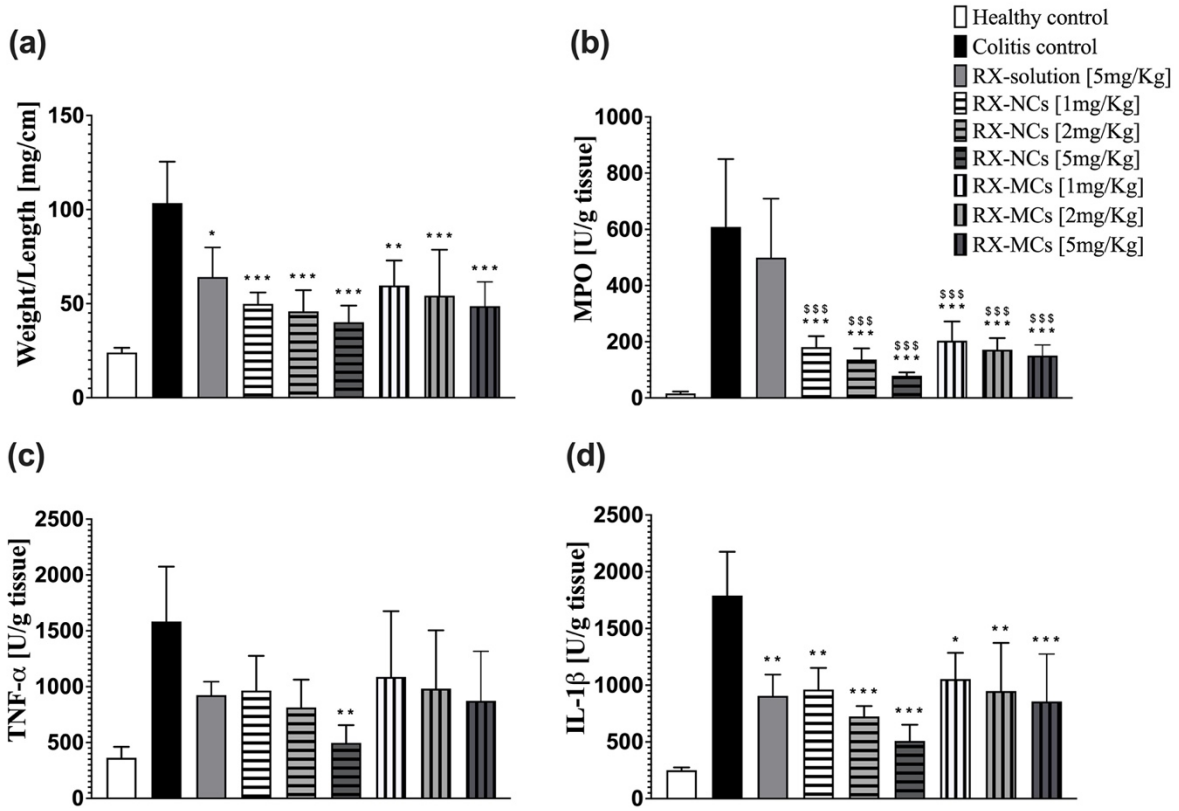

Figure 4-7. (a) Wet colon mass index, (b) MPO levels, (c) TNF-α levels, and (d) IL-1β levels of RX solution, NCs, and MCs treated groups at different doses.
***, * *, and * * * indicate a statistically significant difference compared to colitis control group (* for p<0.05, * * for p<0.01, and * * * for p<0.001).**
$ $ $ indicates a statistically significant difference compared to RX-solution (5mg/Kg) group (p<0.001).

Upon evaluation of the inflammatory parameters of the colitis groups treated with 5 mg/kg RX-NCs of different surface charges and solution, it was found that anionic NCs were the only ones that resulted in a significant decrease in the measured inflammatory parameters

in most cases. While the CAS values of all treated groups were lower than those of the colitis group, anionic NCs caused the most significant difference (Figure 4-8).

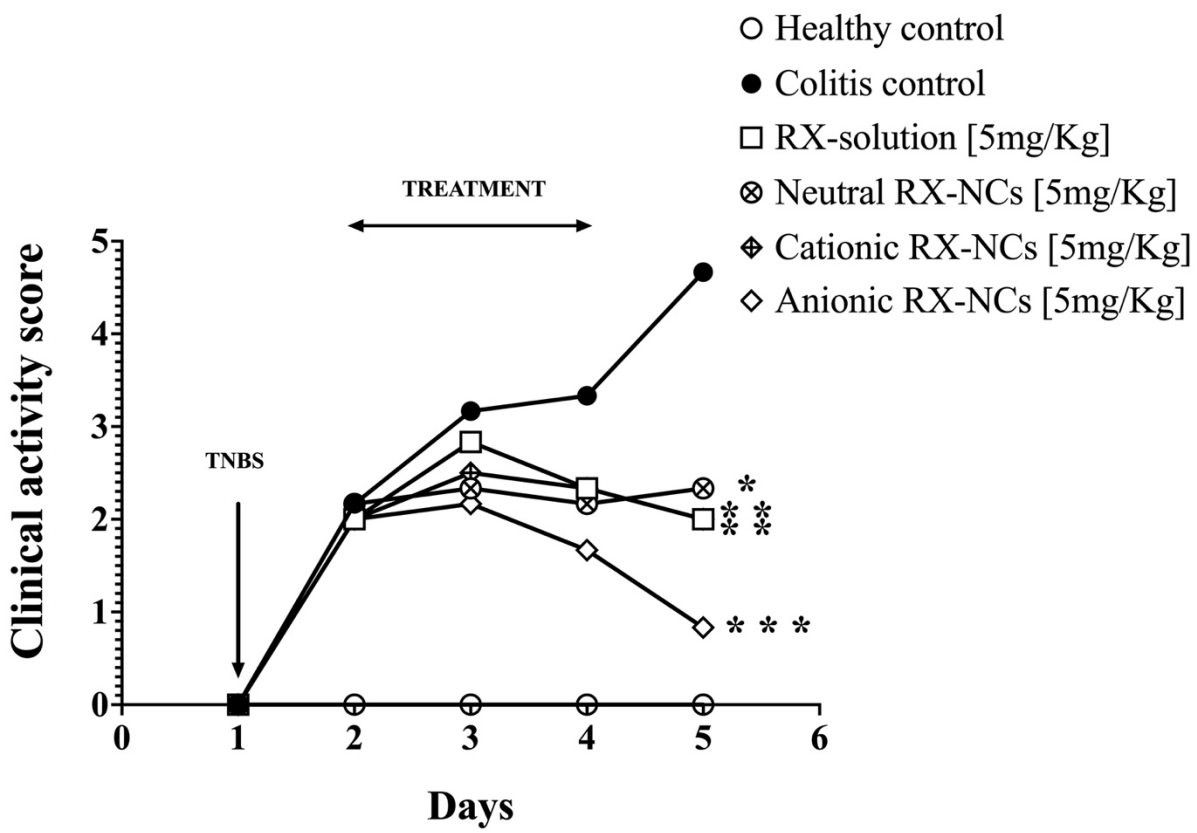

Figure 4-8. Clinical activity score of groups treated with RX solution and nanocrystals of different surface charge (Error bars removed for clarity).

***, * * and * * * indicate a statistically significant difference compared to colitis control group (* for $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$)**

The weight/length ratio was lower in all treated groups than in the colitis group. However, there was no significant difference between them (Figure 4-9 a). Anionic NCs were the only formulation that caused a decrease in MPO levels, which was significantly lower than all other formulations, indicating their stronger effect (Figure 4-9b). Assessment of cytokine levels reinforced these findings: Although all treated groups had lower IL-1β levels (Figure 4-9d), the anionic NCs were the only formulation that produced a decrease in TNF-α levels (Figure 4-9c).

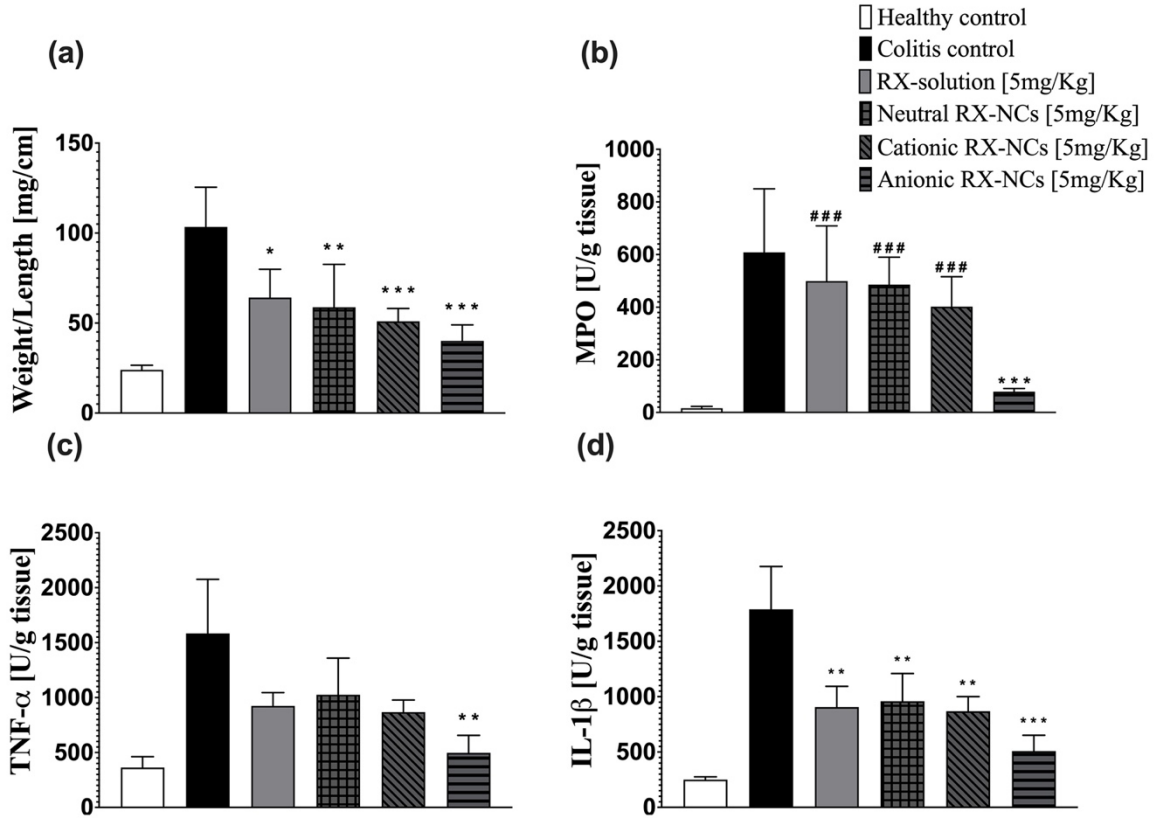

Figure 4-9. (a) Wet colon mass index, (b) MPO levels, (c) TNF-α levels, and (d) IL-1β levels of groups treated with RX solution and nanocrystals of different surface charge.

***, * *, and * * * indicate a statistically significant difference compared to colitis control group (* for $p<0.05$, * * for $p<0.01$, and * * * for $p<0.001$).**

 # # # indicates a statistically significant difference compared to anionic RX-NCs (5mg/Kg) group ($p<0.001$).

4.1.5 Factor Xa blood levels

The assessment of factor Xa in blood showed that both anionic NCs and MCs produced significantly lower factor Xa levels compared to colitis group, indicating their ability to induce systemic anticoagulation (Figure 4-10), Moreover, the effect of NCs as inhibitor for factor Xa was significantly stronger than solution and MCs.

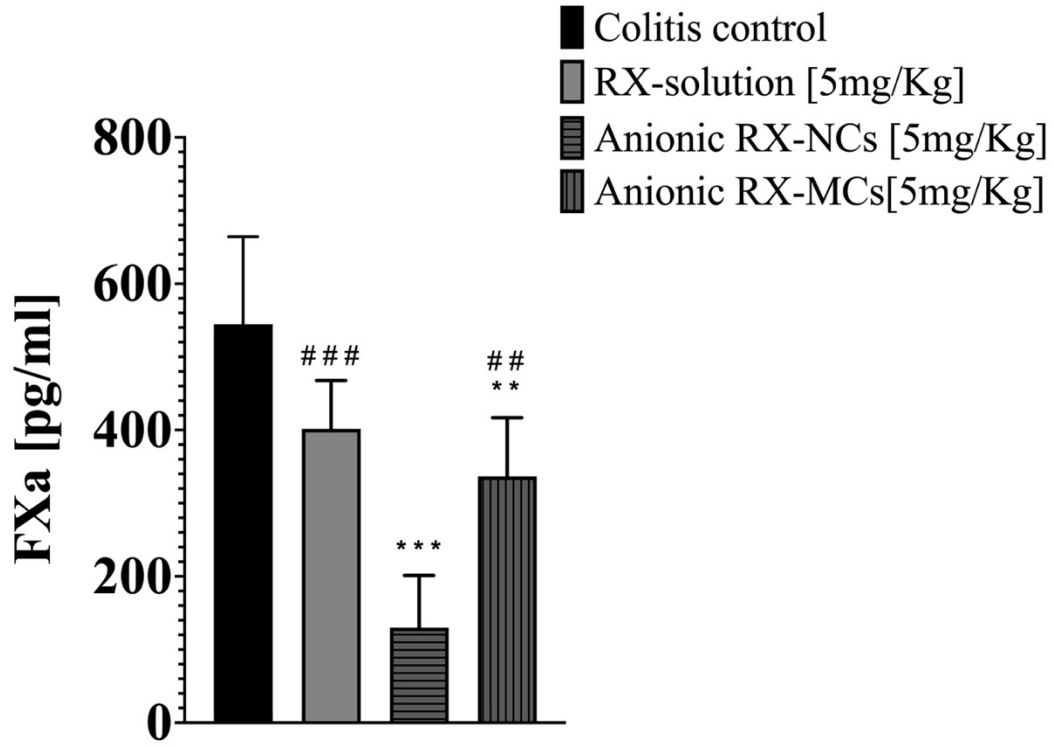

Figure 4-10. The concentration of Factor Xa in blood of mice 15 hours after the rectal administration of RX solution, micro- and nanocrystals.

****, and *** indicate a statistically significant difference compared to colitis control group (** for p<0.01, and * * * for p<0.001).**

 # #, and # # # indicate a statistically significant difference compared to anionic RX-NCs (5mg/Kg) group (# # for p<0.01, and # # # for p<0.001).

4.2 Discussion

IBD is usually associated with a state of hypercoagulability and clot formation within the inflamed bowel and in extra-intestinal tissues. This interdependence of inflammation and blood coagulation has allowed for the development of novel therapeutic approaches for IBD relying on the inhibition of the coagulation pathway e.g., low molecular weight heparin (Pellequer, Meissner, Ubrich, & Lamprecht, 2007; Yazeji et al., 2017). Therefore, we tested RX in TNBS-induced colitis model, and it was able, in most of the cases, to reduce the clinical signs and the markers of inflammation. Oral administration of RX may lead to its systemic absorption, resulting in a reduction of factor Xa throughout the body. This can lead to bleeding disorders and hemorrhagic and non-hemorrhagic side effects with a limited effect on colitis treatment. Therefore, RX formulations were administered rectally to enhance local effects and reduce systemic side effects.

The comparable shape and size distribution of Lumogen® yellow NCs to RX-NCs allowed for comparing the bioadhesion of the MCs and NCs to healthy and inflamed tissue. Both MCs and NCs were found to be more adherent to inflamed tissue than to healthy tissue, making them good candidates for passive targeting to inflammatory sites. On the other hand, the fluorescent NCs showed a greater adherence than MCs. Similar results were reported in which fluorescent MCs remained in the lumen of the colon, whereas the nano counterparts were observed in the inflamed tissue of the colon (Date et al., 2018). The limited distribution of polymeric microparticles in colon tissue compared to nanoparticles has been also extensively reported (Hartwig et al., 2021; Lamprecht et al., 2001). Therefore, CLSM images confirm that NCs do adhere in the same way as polymeric NPs. These results suggest that while NCs and MCs can be used as a strategy to enhance the delivery of RX to the inflamed colon, the NCs achieve greater penetration into the tissue. The higher penetrability of NCs with respect to MCs resulted in a better anti-inflammatory effect of NCs compared to MCs at all doses. The reduction profile of the formulations applied was the same for all the inflammatory indicators assessed, i.e., MPO, IL-1β and TNF-α. There was a dose dependent effect and NCs at the highest dose were the only one to produce a significant reduction of all the inflammatory indicators, indicating their superb efficiency.

Many studies have been conducted to determine the impact of surface charge of nanoparticles on their effect in colitis (Hartwig et al., 2021). One of the studies showed that neutral NPs prepared with the surfactant P20 had the highest accumulation compared to anionic and cationic NPs (Wachsmann et al., 2013). Another study showed that cationic NPs

accumulated the most in inflamed colon tissue (Iqbal et al., 2018b). In our study, negatively charged nanoparticles showed a higher effect in controlling inflammation, as evidenced by the lower levels of MPO, TNF-α, and IL-1β in the group treated with anionic NCs and MCs. Normal colon tissue is covered by mucin, which is negatively charged, suggesting that positively charged NCs adhere to colon tissue longer than their negatively charged or neutral counterparts. However, intestinal inflammation leads to a depletion of mucin in the colon mucosa. In addition, the inflamed tissue is flooded with positively charged proteins, e.g., antimicrobial peptides, transferrin, and permeability-enhancing proteins, so anionic NCs would adhere longer to the inflamed colon mucosa and penetrate it more effectively (Yan et al., 2020).

To better compare the anti-inflammatory effects of the different sized and charged NCs, we measured their ability to reduce the activation of transcription factor NF-κB, which is responsible for the release of inflammatory mediators (T. Liu, Zhang, Joo, & Sun, 2017), through the measurement of the amount of its descendant in inflammation pathway SEAP. Surprisingly, our results showed that the surface charge of the particles had a greater effect than their size. Both anionic MCs and NCs caused a comparable reduction in SEAP amount (about 54%), which was higher than the slight reduction caused by cationic or neutral NCs. These results suggest that the superior effect of anionic micro- and nanocrystals is not only due to their stronger adhesion but also to their higher ability to reduce NF-κB activation.

Although the local effect of RX as anticoagulant in colitis was not prominent as evidenced by the lower values of CAS in all RX treated groups than colitis control, factor Xa level in blood was measured to distinguish between RX solution, anionic MCs, and NCs based on the systemic effect of RX. MCs administration resulted in about the same factor Xa levels in blood as RX solution. However, administration of RX-NCs resulted in lower levels of factor Xa. These results suggest that the NCs have a higher ability to penetrate the mucosa and submucosal tissue and enter the blood than the RX-MCs. Since the systemically absorbed NCs can lead to an anticoagulant effect, anionic RX-NCs may play two roles in this context. They are more efficient in inhibiting inflammation in colon tissues and may exert prophylactic anticoagulant effects to protect patients from the high risk of thrombosis and mortality associated with IBD (Andrade et al., 2018). From these findings, it can be postulated that RX-NCs can be reserved for severe colitis cases associated with hypercoagulation, while anionic RX-MCs can be used for mild or moderate cases.

The results of this study show that anionic RX-NCs have a broader tissue distribution, ana a slight better anti-inflammatory effect than anionic RX-MCs. However, they were strongly

more effective than solution, cationic NCs and non-ionic NCs. Within this context, NCs can be proposed, as other NP, as an important technique to enhance the delivery of drugs to inflamed colonic tissue. Adding to that the low cost of the nanomilling technique used to produce NCs and the superb drug loading and stability of NCs, they are a good candidate to replace current conventional treatment to IBD.

5 Hemin nanoneedles

5.1 Results

5.1.1 Characterization of Hemin NCs

The particle size of the prepared NCs was found to be 155.9 ± 2.5 nm and the polydispersity index was 0.331 ± 0.031. SEM images showed that the NCs have a needle shape and nearly monodisperse (Figure 5-1). Zeta potential was -27.1 ± 5.6 mV.

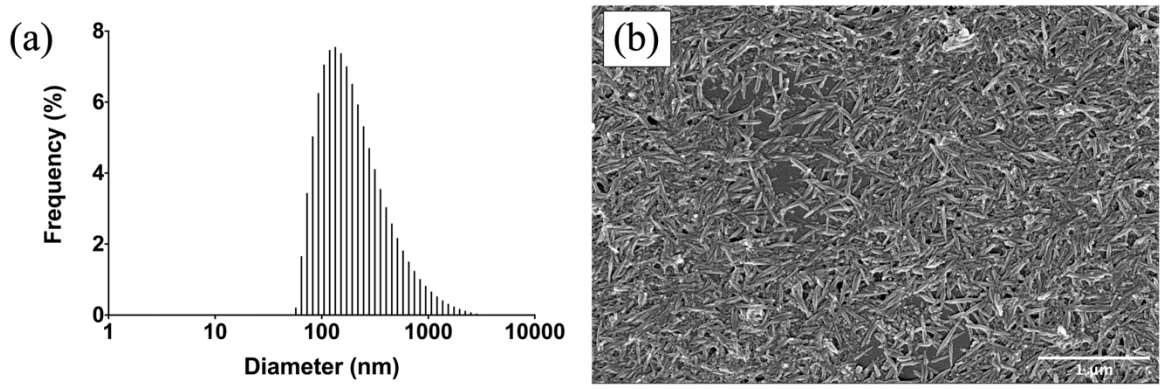

Figure 5-1. (a) Particle size distribution and (b) SEM image of the prepared Hemin NCs (scale bar: 1µm)

5.1.2 In vitro cytotoxicity of hemin formulations

MTT assay showed that hemin NCs are less toxic to macrophage cells compared to hemin solution. LC_{50} of hemin NCs was 13.3 ± 1.5 µg/ml which was more than double the LC_{50} of hemin solution (5.2 ± 1.1 µg/ml). It was also found that hemin concentrations ≤ 3µg/ml as a solution and ≤ 10µg/ml as NCs resulted in more than 70% cell viability (Figure 5-2). Therefore, they were selected for the subsequent in vitro experiments.

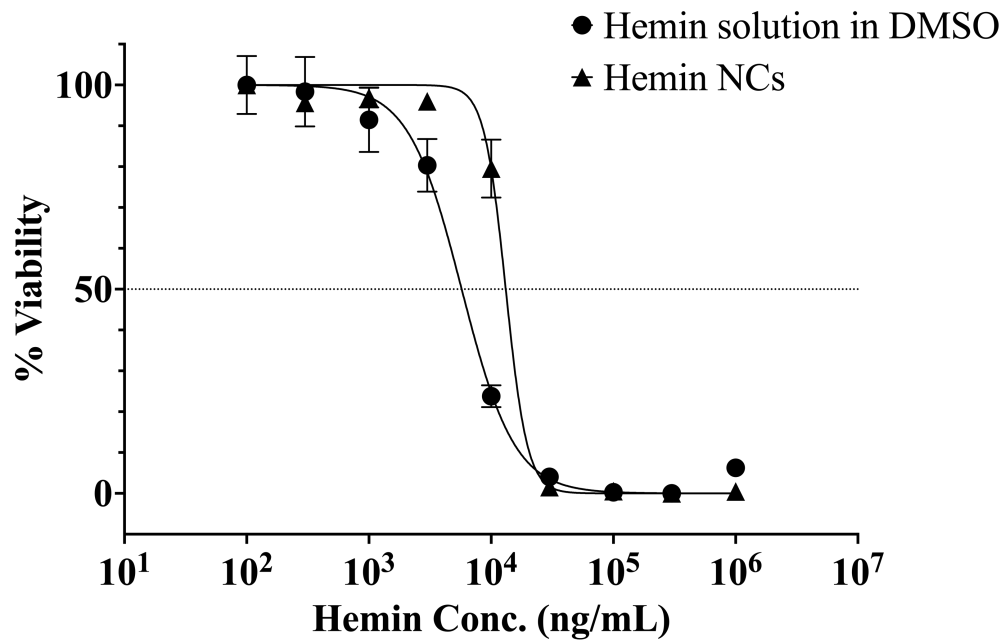

Figure 5-2. Cell viability determined by MTT assay after application of hemin solution in DMSO and hemin NCs for 24 hr.

5.1.3 In vitro Anti-inflammatory effect of hemin formulations

After testing the hemin solutions and NCs on LPS-activated J774-DUAL™ cells, the results showed that after 4 hours there was almost no effect of both solution and NCs. After 12 hours, the hemin solution started to produce a pro-inflammatory effect at lower concentrations, which changed to no effect when the concentrations of hemin were increased. On the other hand, hemin NCs did not produce any pro-inflammatory effect and produced anti-inflammatory effect at the highest applied concentration. After 24 hours, the hemin solution produced a pro-inflammatory effect at lower concentrations, which changed to no effect then anti-inflammatory effect by increasing the concentrations of hemin. On the other hand, hemin NCs produced a significant anti-inflammatory effect at all concentrations, which increased by increasing the concentration of the applied NCs suspension. It was also noticed that even at hemin concentrations where solutions and NCs produced anti-inflammatory effects, hemin NCs produced a significantly higher anti-inflammatory effect (higher value of percent inhibition of SEAP) than hemin solutions (Figure 5-3).

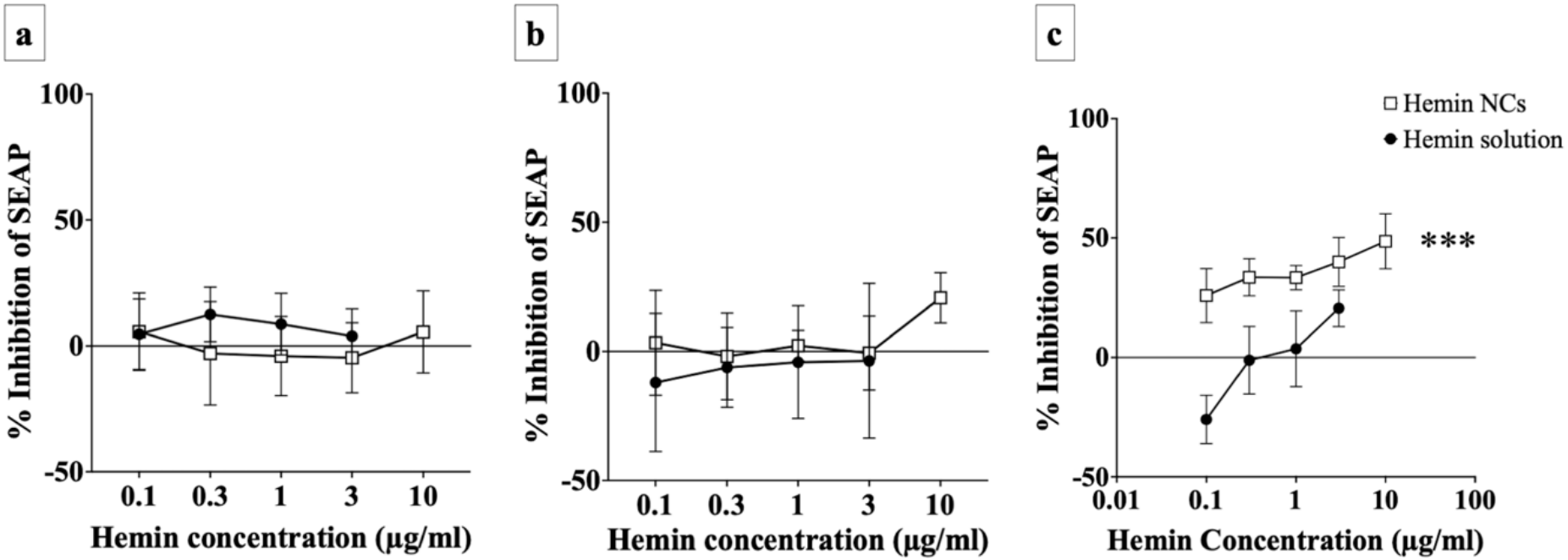

Figure 5-3. The anti-inflammatory effect of hemin NCs and hemin solution on LPS activated J774-DUAL™ cells after (a) 4 hours, (b) 12 hours, and (c) 24 hours.
***** Indicates a statistically significant difference in comparison to hemin solution at each concentration as $p<0.001$**

Application of the hemin solution and NCs to inactivated macrophage cells resulted in no change in the amount of SEAP produced by the cells. There was no difference in SEAP amount between the untreated and treated cells and there was no difference in SEAP amount produced in solution and NCs groups.

After inhibition of the TLR4 receptor using CLI-095, increasing the concentration of the NCs resulted in a higher SEAP amount production. However, increasing the concentration of the hemin solution resulted in a non-significant change in SEAP amount production (Figure 5-4).

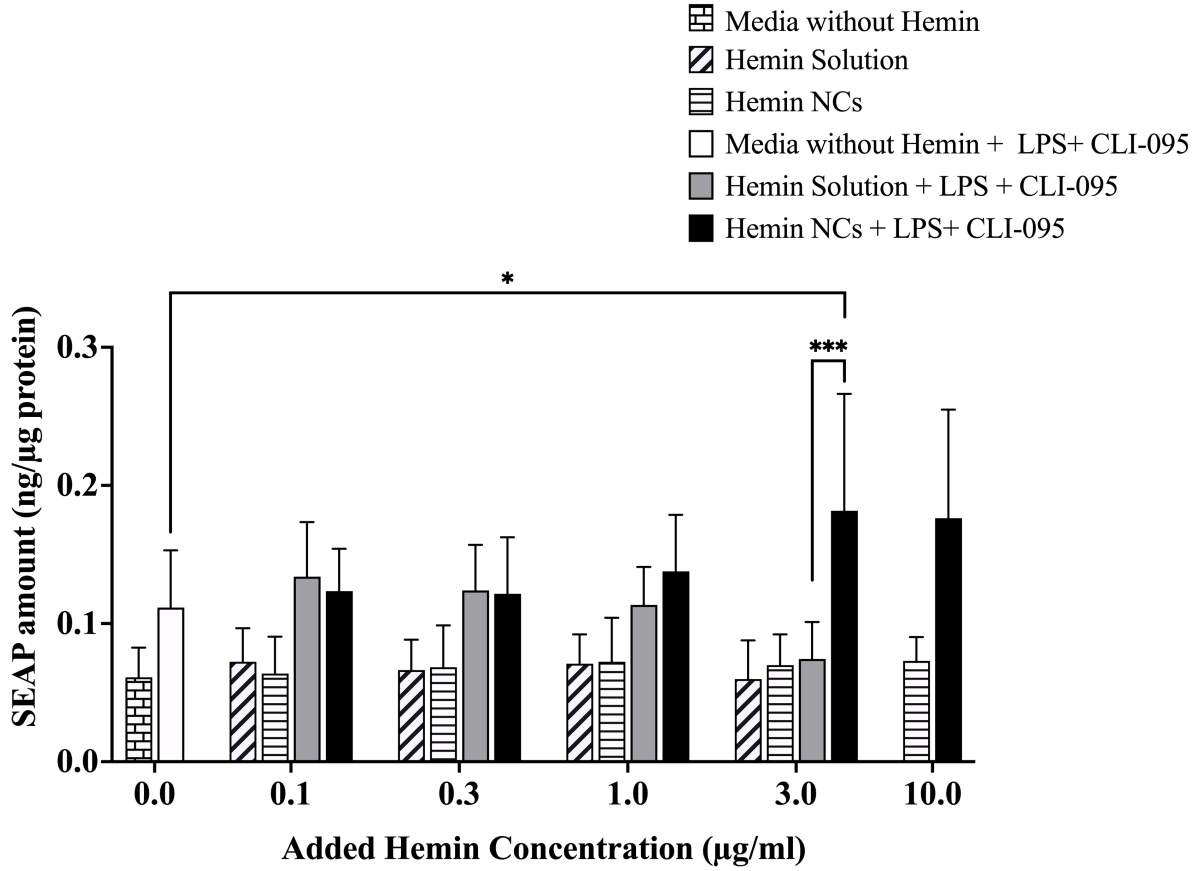

Figure 5-4. SEAP amounts produced after the application of hemin solution and NCs on inactivated and LPS activated TLR4 receptor inhibited J774-DUAL™ cells.
*** and *** Indicates a statistically significant difference as $p<0.05$ and $p<0.001$, respectively.**

5.1.4 Anti-inflammatory effect of hemin in experimental colitis

After evaluation of the clinical activity score, it was found that on day 5 hemin NCs (10mg/Kg) was the only group to produce CAS values that are significantly lower than that of the colitis control group. (Figure 5-5).

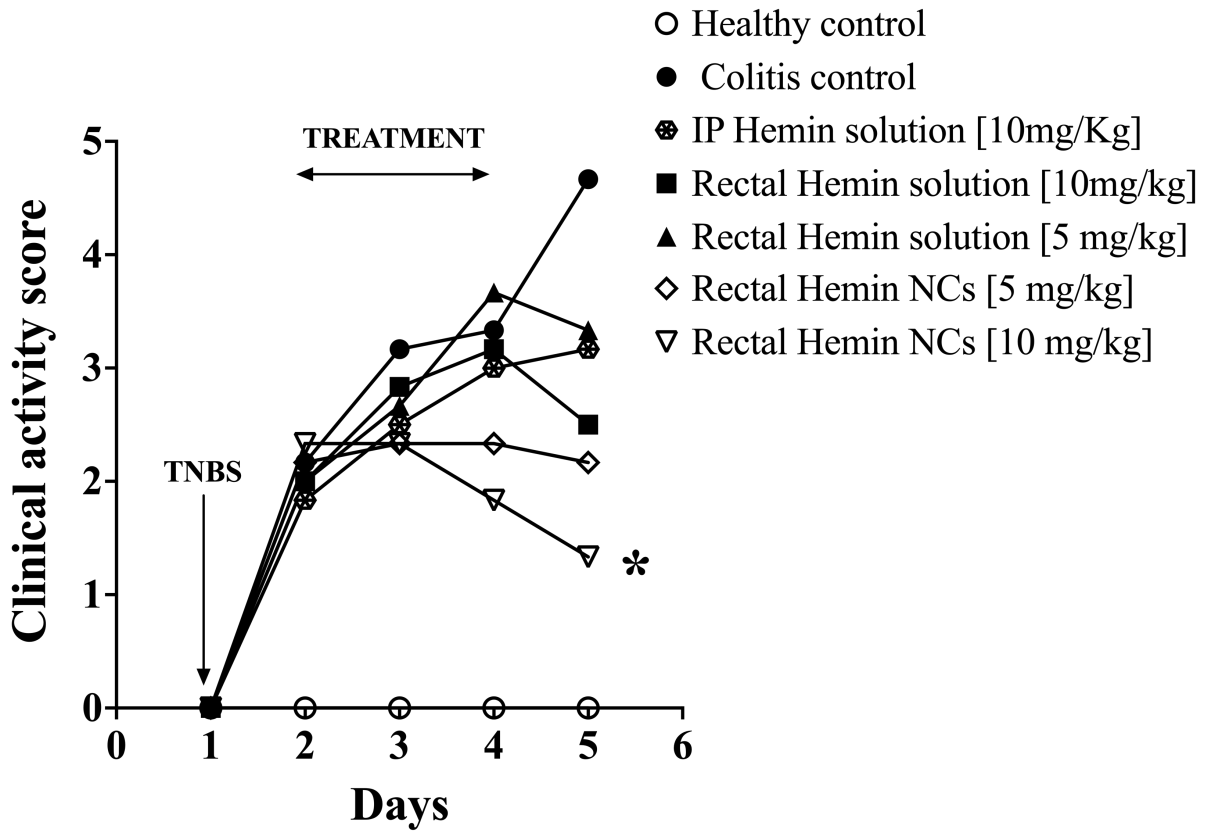

Figure 5-5. The effect of the tested hemin solutions and nanocrystals on the clinical activity score (error bars were removed for clarity).
*** Indicates a statistically significant difference in comparison to the colitis control group ($p<0.05$).**

The weight to length ratio was significantly reduced in all treated groups than in the colitis control group. However, there was no significant difference among the differently treated groups (Figure 5-6).

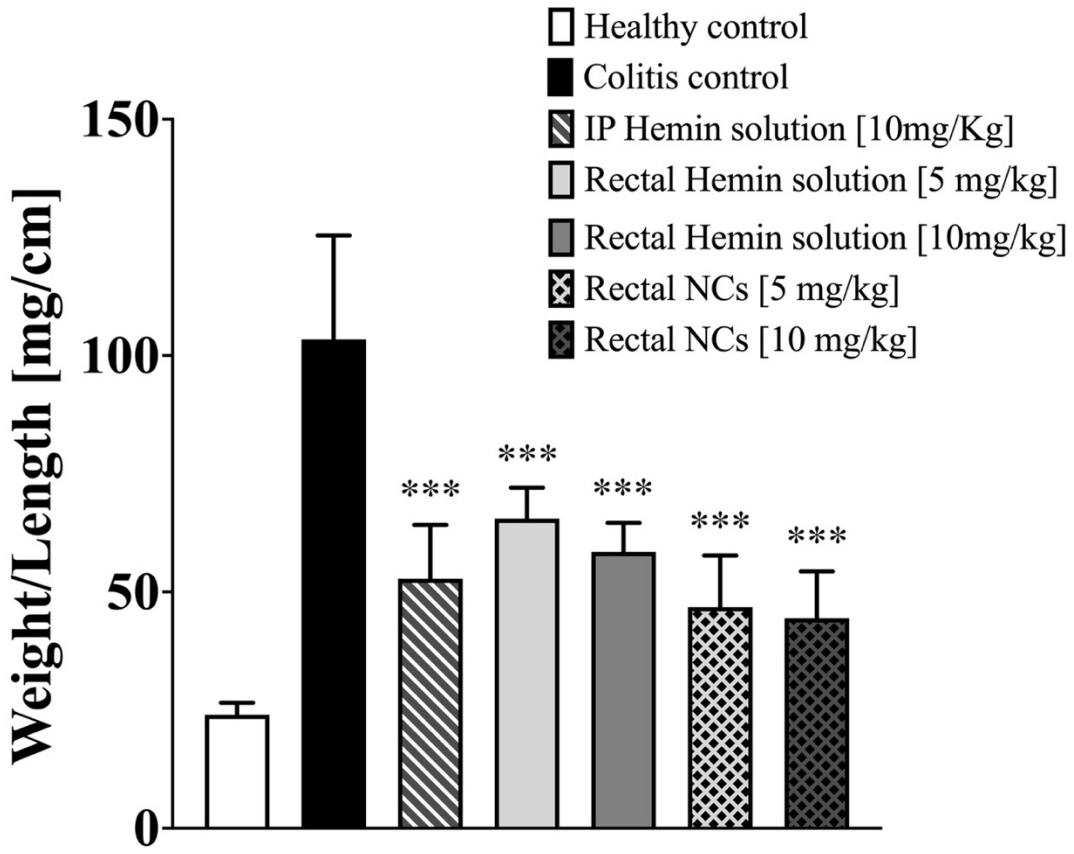

Figure 5-6 The effect of the tested hemin solutions and nanocrystals weight to length ratio of the colon.
***** Indicates a statistically significant difference in comparison to the colitis control group ($p<0.05$).**

In our experiments, all the rectally administered hemin formulations resulted in a significant reduction in MPO levels, and this demonstrates that the rectal application of the formulation shows a more beneficial effect than the intraperitoneal administration which is due to local action where a high amount of the drug is available at the inflammation site. Hemin NCs at a dose of 10 mg/Kg had the strongest effect on the reduction of the MPO amount, as evidenced by having the highest level of significance in difference compared to colitis groups. Moreover, it was the only rectal formulation that significantly reduced MPO levels compared to IP administration of 10 mg/kg hemin solution (Figure 5-7).

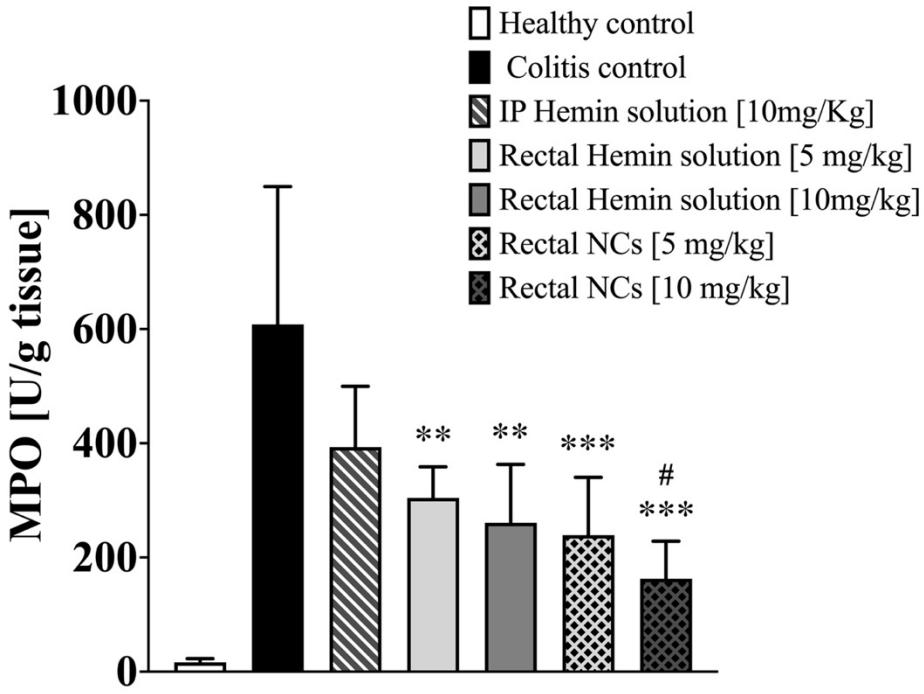

Figure 5-7. The effect of the tested hemin solutions and nanocrystals on Myeloperoxidase tissue concentration.
**** and *** indicate a statistically significant difference in comparison to the colitis control group (** for p<0.01and *** for p<0.001).**
 # Indicates a statistically significant difference in comparison to intraperitoneal hemin solution group at10 mg/Kg (p<0.05).

TNF-α levels showed that only the high doses of hemin solution and NCs (10mg/Kg) showed a significant reduction. On the contrary, hemin NCs of both doses showed a significant decrease in IL-1β levels, while the rectal solution at 10mg/Kg only produced a significant effect (Figure 5-8).

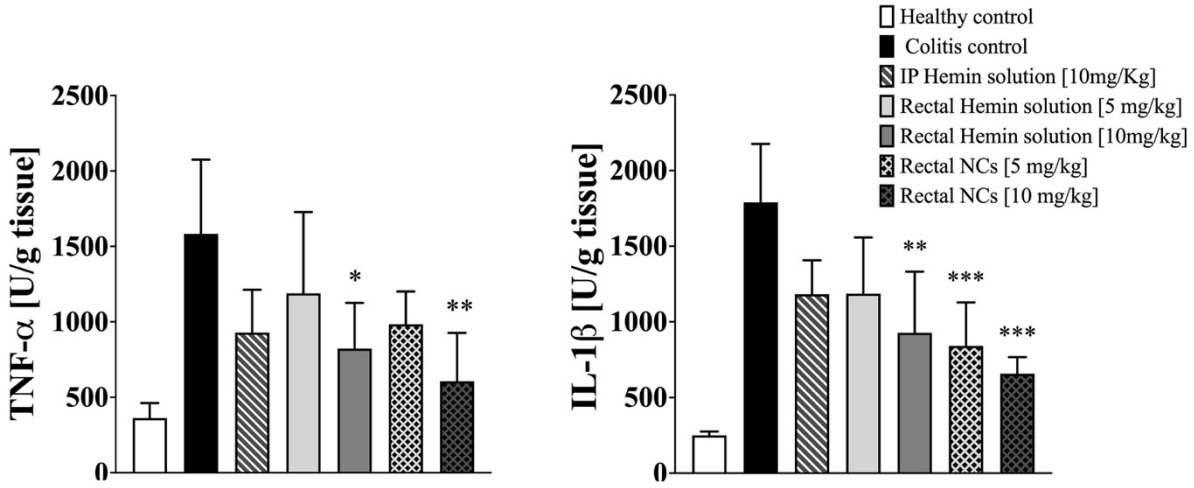

Figure 5-8. The effect of the tested hemin solutions and nanocrystals on cytokines tissue concentration.
***, **, and *** indicate a statistically significant difference in comparison to the colitis control group (* for p<0.05, ** for p<0.01, and *** for p<0.001).**

5.2 Discussion

Hemin has been investigated as an anti-inflammatory agent for the relief of IBD, using hemin as a solution and via IP injection (Kayama et al., 2018; Mateus et al., 2018; Wu et al., 2020; Zhong et al., 2010). However, hemin per se is a two-faced molecule with high toxicity and pro-inflammatory properties (Pradhan et al., 2020). Therefore, the development of a less toxic hemin formulation and with a higher anti- inflammatory effect would be of great value for better management of IBD.

NPs have been extensively used for the passive targeting of drugs to the inflamed colon tissues. However, NPs fabricated from polymers or lipids have limited drug loading capacity, lower stability, and suffer from early drug leakage. Therefore, NCs formulation was selected due their ability to deliver the highest amount of drug per particle compared to any other NPs and their higher stability.

Hemin molecule is known to induce cytotoxicity (Igarashi & Watanabe-Matsui, 2014; Pradhan et al., 2020). However, in our study, the formulation of hemin as NCs showed lower cytotoxicity as indicated by the higher LC_{50} of hemin NCs than double the LC_{50} of hemin solution. This also allowed for the use of higher concentrations of NCs in cell culture experiments.

Moreover, the difference in particle size and PDI between the results of DLS and SEM can be attributed to the needle shape of the NCs. DLS correlates the light scattering it receives and the speed of Brownian motion of the particles with their particle size (Stetefeld, McKenna, & Patel, 2016). Since a different scattering from the tips and sides of the needles is produced, a higher PDI and inaccurate particle size are resulted. Therefore, the values from SEM should be of better accuracy, and monodispersed needle shaped NCs can be assumed.

Previous studies showed that the particle shape of NPs affects their ability for passive targeting, especially to the colon where the tissues are covered with a layer of mucin (Bao et al., 2020b; Barua et al., 2013; Yu et al., 2016). Most of them suggested that nanorods are the most appropriate morphology to be used for the management of IBD. This may be due to the high aspect ratio of nanorods. whereas a nanosphere's diameter governs its size, nanorods can be designed relatively thin but sufficiently long to jiggle and pass through the mucus layer and the defective lining of the colon (Bao et al., 2020b). Likewise, hemin nanoneedles exhibit a high aspect ratio which allows them to penetrate and produce an anti-inflammatory action. In our study, the produced anti-inflammatory action was even higher than that of the solution

which supports that needle-shaped NPs can exert a better penetration and passive targeting than solution.

Hemin has two opposing effects in the context of inflammation. Hemin is a part of Hemoglobin whose presence in tissue signals tissue injury and blood extravasation from capillaries. Therefore, heme is considered as one of the damage/danger-associated molecular patterns (DAMPs) (Gladwin & Ofori-Acquah, 2014; Pradhan et al., 2020). DAMPs can bind to Toll-like receptors (TLRs) and trigger an inflammatory cascade involving activation of the transcription factor Nuclear factor-κB (NF-κB) (L. Chen et al., 2018). However, metabolism of heme intracellularly by Heme Oxygenase-1 (HO-1) results in the production of carbon monoxide (CO), Iron, and biliverdin. Biliverdin and CO have anti-inflammatory and antioxidant effects (Motterlini & Foresti, 2014; Pradhan et al., 2020; Ryter, Alam, & Choi, 2006). In our experiments, the hemin solution at lower concentrations resulted in a pro-inflammatory effect, as evident from the negative value of the percent inhibition of SEAP. On the contrary, hemin NCs resulted in an anti-inflammatory effect at the same concentrations and higher anti-inflammatory effect at higher concentrations.

After testing the formulations on inactivated cells, SEAP levels did not change indicating that these formulations could not induce an inflammatory response in the normal macrophage cells, i.e., those not activated by inflammation. In addition, there was a difference in the effect of the NCs on activated macrophage cells with and without TLR4 receptor inhibition. In TLR4 receptor-inhibited cells, SEAP levels increased with increasing concentration of the hemin NCs used, which was not the case in activated macrophages without TLR4 inhibition. This suggests that when the TLR4 receptor is inhibited, the anti-inflammatory effect of the hemin NCs is impaired, which may indicate a role for the TLR4 receptor in the anti-inflammatory effect of the NCs. Moreover, another inflammatory pathway induced by hemin NCs might be activated to compensate for the inhibition of TLR4 receptor. These findings suggest further studies to understand the complex mechanism of hemin in inhibiting inflammation.

Upon evaluation of the inflammatory parameters measured in experimental colitis, it can be noticed that intrarectal hemin NCs at 5 mg/kg produced comparable or slightly greater reduction of the levels of MPO, TNF-α, and IL-1β than hemin solution at 5 and 10 mg/kg. Besides, hemin NCs at a dose of 10 m/kg was even a better formulation, as evident from its ability to improve clinical signs of the disease, as indicated by the low CAS value, and

significantly reduce inflammatory mediators levels, e.g., MPO, TNF-α, and IL-1β, in comparison to those of colitis control.

Although NCs technology can be considered a subclass of NPs, they outperform their counterparts in their ability to transport larger amounts of drug with minimal leakage and higher stability. The unique particle morphology for each drug NCs that is produced after the drug is milled, which depends on the crystal structure of the drug, has made it possible to study the ability of nanoneedles to deliver drugs better than a solution. Additionally, since the NCs are easy to fabricate and scale, our study suggests that hemin- NCs are a good substitute for conventional therapies based on drug dissolution, as well as for polymeric and lipid-based NPs for the treatment of IBD.

6 Conclusion and outlook

This study showed that the most important factors in fine-tuning the size of the nanocrystals produced were the concentration of the stabilizer solution, the diameter of the beads, and the milling time. The level and effect of these factors varied from one study to another. Therefore, there are no optimal values that can be generally applied, but they should be specifically evaluated for each study. The production of NCs by milling demonstrated in this study is quite simple and allows for consistent scale-up of this method, typically by large-scale mills or homogenizers, without the many anticipated problems associated with large-scale production of polymer- or lipid-based NPs. Since no organic solvents are used in the top-down approach, the formulations are environmentally friendly and exhibit a better safety profile.

Application of this NCs formulation to drugs such as RX or Hemin increased their anti-inflammatory effect over the respective solution at the same dose. The study also showed that the effect of particle size of NCs is not as strong as the surface charge of NCs. This was evidenced by the stronger anti-inflammatory effect of anionic NCs over non-ionic and cationic counterparts in vitro and in vivo. However, anionic RX NCs had a slightly stronger local anti-inflammatory effect than anionic MCs. Anionic NCs showed a stronger systemic reduction of factor Xa than MCs, suggesting that the use of NCs can be reserved for severe colitis cases associated with hypercoagulation, whereas anionic RX MCs can be used to treat mild or moderate cases. The study suggests that RX NCs have higher therapeutic value in IBD than polymeric or lipid-based NPs due to their high drug loading, higher stability, and ease of preparation and scaling.

Hemin nanoneedles exhibit lower cytotoxicity and stronger anti-inflammatory activity than a solution of hemin in vitro and in vivo. Therefore, a lower dosage and safer formulation of hemin can be used to treat IBD when formulated as NCs than in a solution. Despite the irregular shape of NCs and even with a high aspect ratio as in nanoneedles, NCs, like other NPs, can enhance the effect of the formulated drug over the drug solution. Further studies are certainly needed to clarify whether NCs can be used as a general strategy to enhance drug efficacy in IBD or whether their effect is limited to specific actives.

Our results suggest a promising effect of rectally administered NCs. An important next step is certainly the formulation of NCs in a secondary vehicle for oral administration. The oral formulation of hemin based NCs is better suited to reach the inflamed colon tissue throughout

the gastrointestinal tract, which is of great benefit in the inflammatory lesions associated with Crohn's disease. In this context, NCs are of greater value than a hemin solution because they can accumulate in these patches and exert their inflammatory effects. RX-NCs are a better candidate than heparin for oral therapy of IBD. While the latter is a protein that can be enzymatically degraded on its way through the gastrointestinal tract and in the colon, RX-NCs are able to withstand the harsh conditions of the gastrointestinal tract and reach the colon to exert their effects. Therefore, heparin should mostly be formulated as polymer or lipid-based NPs, which should then be enteric-coated to be protected from the enzymes of the gastrointestinal tract. Polymer or lipid-based NPs suffer from low drug loading, early drug leakage, low stability, and high production cost. On the other hand, NCs are composed mainly of the active ingredient, which make them a good candidate for oral solid dosage forms, e.g., tablets, capsules, or powders.

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8 Summary of the used publication

Olga Hartwig, Maryam A. Shetab Boushehri, Karim S. Shalaby, Brigitta Loretz, Alf Lamprecht, Claus-Michael Lehr, 2021. Drug delivery to the inflamed intestinal mucosa - targeting technologies and human cell culture models for better therapies of IBD. Adv. Drug Deliv. Rev. 113828 (2021) doi:10.1016/j.addr.2021.113828.

This article provides a comprehensive overview of strategies to combat the increasingly occurring inhibitory bowel disease (IBD), which include many avenues of research. First, the development of new drugs and biologics that can not only relieve symptoms but also reverse the inflammatory state on a mechanistic approach and prevent the recurrence of IBD. Second, these drugs must be intelligently delivered to the sites of inflammation and prevent off-target delivery to avoid adverse effects or premature release of the drug before it reaches the target. Therefore, the drug requires a carrier to perform the task of targeting. This carrier can be of versatile origin, originating from biology or nanotechnology, and can make its targeting ability dependent on its physicochemical properties or the inflammatory environment. These approaches are accompanied by the development of in vitro and in vivo models that can mimic the disease state with its macro and microenvironment to allow testing of the developed drugs or carrier systems.

The pathophysiology of IBD was subsequently discussed, followed by the preclinical IBD models for drug development, as the development of new drugs and delivery systems would be misled if applied to an inefficient colitis model. Therefore, the continuous development of animal models and cell- and tissue-based in vitro models was thoroughly discussed, including chemically induced models and genetically engineered models for the former and traditional 2D gut in vitro models, advanced 3D co-cultures - scaffold-free or ECM-based, culture techniques for gut organoids, ex vivo tissue cultures, microfluidic devices (gut-on-a-chip) and microbiome co-culture systems for the latter.

Drug treatment of IBD has been and still is dependent on reducing the inflammatory state and treating the symptoms. For decades, mesalazine/5-ASA was used for mild or moderate colitis, and corticosteroids or immunosuppressants were used in severe cases. However, progressive research and understanding of the pathophysiological mechanisms of IBD led to the development of more specific drugs that can break the vicious cycle of inflammation by neutralizing one of the inflammatory mediators, inhibiting leukocyte adhesion, or inhibiting the transcription factors that lead to the synthesis of the inflammatory cytokines. Therefore, the landscape and pipeline of IBD therapeutics were then discussed, including conventional and

current medical interventions, novel IBD agents in clinical trials, and alternative IBD therapies such as microbiota manipulation and stem cell therapy.

Numerous delivery systems of different origins have been used for the increasingly new drugs against IBD. They are divided into biological and nanotechnology drug delivery systems according to their origin. In the fourth section of this review article, biological drug delivery has been discussed in detail, including bacterial drug delivery, drug delivery from cells and cellular components, extracellular vesicles, and drug delivery from viruses. In this context, biosafety and containment of these biological systems as well as their inducibility and reactivity were also discussed.

This was followed by the section on nanotechnology-based drug delivery (the doctoral student's contribution). This section discussed nanoparticles (NPs) that depend on the use of a non-living, biodegradable carrier. NPs can be fine-tuned in size, surface charge, and shape to achieve better accumulation in inflamed tissue than in non-inflamed tissue. Furthermore, the carrier material can serve additional functions, such as physically retarding the release of drug from the NPs or releasing the drug on demand when exposed to environmental changes in the inflammatory state. In addition, the NPs can be decorated with ligands or antibodies against specific targets that are actively targeted to the inflammatory site. All the above factors can be combined to form multifunctional NPs consisting of a core formed by a carrier and an active agent, both of which have an anti-inflammatory effect. This core is covered with a functional shell of a stimuli-responsive polymer, i.e., a pH-sensitive or oxidative stress-responsive polymer and decorated with a ligand or an antibody to target specific molecules or cells in the inflamed colon tissue.

Systems based on biology or nanotechnology must ultimately be formulated into a dosage form that can be administered by the patient. Therefore, the last section of the review discussed the challenges and advances in translating these delivery systems into formulations and the potential routes of administration for these formulations.

9 Publications

9.1 Articles

Hartwig, O., Shetab Boushehri, M. A., Shalaby, K. S., Loretz, B., Lamprecht, A., & Lehr, C. M. (2021). Drug delivery to the inflamed intestinal mucosa – targeting technologies and human cell culture models for better therapies of IBD. Advanced Drug Delivery Reviews, 175, 113828. https://doi.org/10.1016/J.ADDR.2021.113828

Shalaby, K. S., Ismail, M. I., & Lamprecht, A. (2021). Cyclodextrin Complex Formation with Water-Soluble Drugs: Conclusions from Isothermal Titration Calorimetry and Molecular Modeling. AAPS PharmSciTech, 22(7), 1–9. https://doi.org/10.1208/s12249-021-02040-8

9.2 Abstracts (Conference participation):

Shalaby, K. S., Moulari B., Béduneau A., Pellequer Y. & Lamprecht, A. (2020). Hemin nanocrystals for the management of Inflammatory bowel disease. On-demand Talk at CRS 2020, Virtual Conference, 29th June to 2nd July

Shalaby, K. S., Ismail, M. I., & Lamprecht, A. (2020). Isothermal Titration Calorimetry And Molecular Modelling Study Of Cyclodextrin Complexation: The Effect Of Guest Flexibility. Poster presentation at CRS 2020, Virtual Conference, 29th June to 2nd July.

Shalaby, K. S. & Lamprecht, A. (2019). Illustrative and Predictive Tools: Isothermal Calorimetry Combined with Molecular Modelling for the Study of Cyclodextrin/Drug Interactions. Poster presentation at AAPS PharmSci 2019, San Antonio, Texas, USA, 3rd to 6th November.