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Nanoparticles-in-thermosensitive enemas as novel rectal anti-HIV microbicides

Dissertation submitted to the University of Coimbra for the degree of Master in Biomedical Engineering

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This work was developed in collaboration with:

"*What's coming will come and we'll just have to meet it when it does." J. K. Rowling*

To my Grandmother.

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Resumo

Os microbicidas de base nanotecnológica poderão desempenhar um papel importante na prevenção na transmissão sexual do VIH. No entanto, apesar do seu potencial, os nanossistemas microbicidas requerem ser incorporados em formas farmacêuticas de modo a constituir produtos tangíveis, nomeadamente para administração retal. Os enemas termossensíveis são formulados como sistemas líquidos à temperatura ambiente mas que sofrem uma transição sol-gel a um pouco menos do que 37 ºC, formando assim sistemas semi-sólidos tais como géis. O seu estado líquido inicial poderá aumentar a distribuição dos nanossistemas ao longo da mucosa logo após administração retal, enquanto que a sua posterior gelificação poderá promover uma permanência prolongada no colorreto. De modo a testar esta possibilidade, foram desenvolvidos neste trabalho enemas termossensíveis baseados em poloxâmero 407 para serem ustilizados como veículos para a administração retal de potenciais nanotransportadores de fármacos microbicidas, nomeadamente nanopartículas (NPs) de ácido poli(láctico-*co*-glicólico) (PLGA). O estudo dos enemas incluiu a determinação das suas propriedades tecnológicas mais relevantes, nomeadamente a temperatura e tempo de transição sol-gel, características reológicas, pH e osmolalidade, bem como ensaios de citotoxicidade *in vitro* através da execução de ensaios de redução do MTT e de libertação da LDH. Um enema termossensível carregado com NPs (*NPs-in-thermo*) otimizado foi ainda testado num estudo preliminar quanto à sua distribuição e retenção *in vivo* num modelo de murganho.

Foram preparadas diferentes formulações de enemas termossensíveis pelo método a frio. A concentração e o lote do poloxâmero 407 utilizados tiveram um grande impacto na transição sol-gel e viscosidade dos enemas, enquanto que a incorporação de excipientes como a glicerina e o ácido sórbico, respectivamente, influenciaram a osmolalidade e o pH. Foi selecionada uma base para enema termossensível otimizada com a composição de 15% (*w/v*) em poloxâmero 407 e 2% (*w/v*) em glicerina num tampão fosfato 10 mM e a pH final de 7,0 para incorporação de NPs de PLGA (170-200 nm) obtidas por nanoprecipitação com concentrações de 0,1 a 1% (*m/v*). Os *NPs-in-thermo*

obtidos possuiam valores de temperatura e tempo de transição sol-gel de 27-31 ºC e 1,6-2,2 min, respetivamente, viscosidade de 0,0021-0,0031 Pa s (20 ºC) ou 2,0-2,6 Pa s (37 ºC) a uma taxa de cisalhamento de 40 s^{-1}, pH de 6,9-7,2 e osmolalidade de 348-470 mOsm/Kg. A citotoxicidade dos *NPs-in-thermo* revelou-se baixa quando determinada em células colorretais Caco-2 e por comparação com produtos lubrificantes comerciais. A análise de imagens de fluorescência no infravermelho próximo, captadas *in vivo* e *ex vivo* em murganhos e os seus respetivos tecidos colorretais, mostrou que NPs fluorescentes incorporadas no enema termossensível à concentração de 0,5% (*p/v*) podem ser localizadas com sucesso após a sua administração retal. Para além disso, os dados preliminares obtidos sugerem que a utilização do *NPs-in-thermo* promove uma menor distribuição inicial mas uma retenção mais prolongada das NPs ao nível colorrectal, nomeadamente quando comparado com a administração das mesmas NPs num veículo líquido (solução tampão fosfato salino a pH 7,0). Em conclusão, o *NPs-in-thermo* otimizado neste trabalho poderá constituir uma plataforma interessante para a administração retal de fármacos microbicidas anti-VIH.

Palavras-chave: Administração retal de medicamentos; Modelo de murganho; Nanomedicina; Poloxâmero; Polímeros termossensíveis.

Abstract

Nanotechnology-based microbicides may play a relevant role in preventing sexual HIV transmission. However, despite their potential, microbicide nanosystems require to be incorporated into suitable dosage forms in order to constitute tangible products, namely for rectal use. Thermosensitive enemas can be designed as liquids at room temperature that undergo sol-gel transition at just below 37 ºC, thus forming semi-solid systems such as gels. Their initial liquid state may enhance distribution of nanosystems throughout the mucosa upon rectal administration, while subsequent jellification may prolong local residence. In order to attest this possibility, thermosensitive enemas based on poloxamer 407 were developed in this work as vehicles for the rectal administration of surrogate microbicide drug nanocarriers, namely poly(lactic-*co*-glycolic acid) (PLGA)-based nanoparticles (NPs). Enemas were studied for relevant technological features including sol-gel transition temperature and time, rheological properties, pH and osmolality, as well as for *in vitro* cytotoxicity using the MTT reduction and LDH release assays. An optimized NPs-in-thermosensitive enema (NPs-in-thermo) was further assessed for *in vivo* distribution and retention in a preliminary study using a mouse model.

Different thermosensitive enema formulations were obtained using the cold method. The concentration and batch of poloxamer 407 had the most impact on sol-gel transition and viscosity, while the incorporation of excipients such as glycerin and sorbic acid impacted osmolality and pH, respectively. An optimized thermosensitive enema base comprising 15% (*w/v*) poloxamer 407 and 2% (*w/v*) glycerin in 10 mM phosphate buffer at a final pH of 7.0 was considered for incorporating 0.01-1% (*w/v*) of PLGA-based NPs (170-200 nm) obtained by nanoprecipitation. The NPs-in-thermo featured mean values of sol-gel transition temperature and time of 27-31 ºC and 1.6-2.2 min, respectively, viscosity of 0.0021-0.0031 Pa s (20 ºC) or 2.0-2.6 Pa s (37 ºC) at a shear rate of 40 s^{-1}, pH of 6-9-7.2 and osmolality of 348-470 mOsm/kg. Cytotoxicity of NPs-in-thermo to Caco-2 colorectal cells was shown to be low, namely when compared to commercial lubricant products. *In vivo* and *ex vivo* near infrared fluorescence imaging of mice and colorectal tissues, respectively, showed that fluorescent NPs incorporated into

thermosensitive enemas at 0.5% (*w/v*) could be successfully tracked upon rectal administration. Moreover, preliminary data suggest that the use NPs-in-thermo mildly reduced initial distribution but prolonged retention of NPs at the colorectum as compared to NPs administered in a liquid vehicle (phosphate buffered saline, pH 7.0). In conclusion, the NPs-in-thermo proposed and optimized in this work may constitute an interesting platform for the rectal delivery of anti-HIV microbicide drug candidates.

Keywords: Mouse model; Nanomedicine; Poloxamers; Rectal drug delivery; Thermosensitive polymers.

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Abbreviations

AIDS: acquired immunodeficiency syndrome;

ASO: antisense oligonucleotide;

AUC: area-under-the-curve;

CEA: carcinoembryonic antigen;

C_{max}: maximum concentration;

CPP: cell penetrating peptide;

Cy7.5: cyanine 7.5;

DMEM: dulbecco's modified eagle medium;

DNBS: dinitrobenzene sulfonic;

DSS: dextran sulfate sodium;

FITC: fluorescein isothiocyanate;

2-AG: 2-arachidonoylglycerol;

Gal-LMWC: galactosylated low molecular weight chitosan;

GIT: gastrointestinal tract;

HIV: human immunodeficiency virus;

IBD: inflammatory bowel disease;

LDH: lactate dehydrogenase;

MSM: men who have sex with men;

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide;

MW: molecular weight;

NIR: near infrared;

NPs: nanoparticles polymeric;

NPs-in-thermo: nanoparticles polymeric in thermosensitive enema;

PEG: polyethylene glycol;

PEO: polyethylene oxide;

PK: pharmacokinetics;

PLGA: poly(lactic-co-glycolic acid);

PPO: polypropylene oxide;

PrEP: pre-exposure prophylaxis;

SLN: solid lipid nanoparticles;

TAG-72: tumor associated glycoprotein-72;

TEM: transmission electron microscopy;

TF: thomsen-friedenreich;

TNBS: trinitrobenzene sulfonic acid;

TNF: tumor necrosis factor;

TPGS: D-α-tocopherol polyethylene glycol 1000 succinate;

UC: ulcerative colitis;

UNAIDS: united nations programme on hiv/aids;

WHO: world health organization.

1. Motivations, objective and document Structure

1.1. Context and motivations

The human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) pandemic remains a huge healthcare challenge of our days [1]. Despite current availability of effective antiretroviral treatment, individuals infected by the virus cannot be cured and become potential sources of new infections. Thus, prevention constitutes a cornerstone in the fight against HIV/AIDS. Various modalities are available, with condom use topping the list. However, and despite steadily decreasing numbers over the last two decades, data from the Joint United Nations Programme on HIV/AIDS (UNAIDS) estimate that around 1.8 million new infections occurred worldwide in 2016 alone, mostly from sexual transmission [2]. Thus, it seems clear that new preventive approaches are needed to tackle new infections [3]. Antiretroviral drug-based strategies, particularly oral pre-exposure prophylaxis (PrEP), have been emerging over recent years, often with considerable success. Yet, problems related with poor adherence, systemic adverse effects and costly regimens have been raised and often limit the wide use of oral PrEP. One alternative is the use of topical antiretroviral drug-based products termed microbicides, which could be used around the time of sexual intercourse, thus preventing infection at initial transmission steps [4]. While vaginal microbicides are in advanced state of development, particularly the dapivirine ring [5], rectal microbicides seem to have been left behind and require more research into their potential usefulness [6]. Nanotechnology-based approaches have been proposed to enhance the safety and efficacy of rectal microbicides [7]. Features such as controlled drug release, targeted drug delivery or modulation of mucoadhesive properties of nanosystems may be achievable by proper engineering of drug nanocarriers and considered as positive for microbicide development. Distribution and retention of drugs in the colorectum is another potential advantage of using drug nanocarriers. However, recent work from our group and others showed that polymeric nanoparticles (NPs) are rapidly eliminated from the colorectum of mice upon rectal administration [8, 9]. Thus, additional strategies for enhancing both colorectal

distribution and retention of potential microbicide drug nanocarriers are needed. One possibility could be the use of liquid vehicles (enemas) for the administration of NPs, which could enhance initial distribution (important for wide coverage of the mucosa and protection against HIV transmission). However, since liquid dosage forms are prone to leakage, a sol-gel transition could be beneficial in order to yield a gel-like system that would favor retention. Body temperature appears to be an excellent trigger for inducing sol-gel transition. It should also be noted that enemas are well accepted by men who have sex with men (MSM) [10], an important target population for rectal microbicides even if women engaging in anal sex could also benefit from such products. Thus, a thermosensitive enema could be a potentially interesting vehicle for the rectal administration of microbicide drug nanocarriers.

1.2. Main objective and specific aims of the work

The main objective of this project is to develop a thermosensitive enema that could serve as a general vehicle for rectal administration of NPs, particularly to be used in the future for establishing anti-HIV microbicide products. The resulting system, further referred to as NPs-in-thermo, should be liquid at room temperature but, upon exposure to body temperature following administration, should jellify. In this way, wide colorectal distribution could be achieved due to the spreading ability of the enema but, also, retention at the colorectum could be promoted upon sol-gel transition of the system. The widely used poloxamers were selected as tentative candidates for developing thermosensitive enemas, while poly(lactic-*co*-glycolic acid) (PLGA)-based NPs were tested as model nanocarriers for microbicide drugs to be incorporated into optimized vehicles.

With the main objective of this work in mind, the following specific aims were established: (i) develop and optimize a simple thermosensitive enema formulation based on poloxamers; (ii) prepare and characterize basic physicochemical properties of PLGA-based NPs, including fluorescently labeled ones, for further incorporation into thermosensitive enemas; (iii) assess the cytotoxicity profile of NPs-in-thermo using a relevant colorectal cell model; and (iv) perform exploratory in vivo experiments in mice

using fluorescently labeled NPs-in-thermo in order to establish the initial conditions for future and more detailed distribution and retention assessments.

It is expected that, at the end of this work, one optimal NPs-in-thermo formulation can be obtained, serving as a potential candidate for further development and establishment of a putative rectal microbicide for preventing sexual transmission of HIV.

1.3. Structure of the dissertation

The present document is organized in two main parts comprising a revision of relevant scientific literature and a following section detailing on the conducted experimental work. After the hereby introduction to the motivations and objective of the proposed work, as well as the document structure, the revision of the state-of-the-art of research involving rectal drug delivery and the potential impact of nanotechnology is presented in section 2. The experimental part of the work is described and critically discussed in sections 3 and 4, corresponding to *"Materials and methods"* and *"Results and discussion"*, respectively. Finally, section 5 presents the main conclusions of this dissertation and prospects for future studies, followed by the list bibliography used as references throughout this document. The last section of the document is the *Annex 1* where are supplementary documents.

2. Revision of the literature[1]

The rectum has been considered as a route for drug administration since the dawn of medicine, with first reports of its use dating back to the times of ancient Egypt civilization (circa 16^{th}-15^{th} century BC) [11]. Rectal drug delivery persisted until our days although mostly restricted to the relief of local conditions (*e.g.*, treatment of hemorrhoids or relief of constipation) or use in particular populations such as infants and children, as well as adults in which the oral route is somehow compromised (*e.g.*, states of unconsciousness, vomiting or nausea) and the elderly. Indeed, poor acceptance by users, which is mostly related with cultural issues, usually impairs the wider use of the rectal route [12]. The field of rectal drug delivery has reemerged over recent years, with substantial advances in the understanding of the colorectal biology and developments in drug dosage form technology being boosted by the increasing interest in anti-HIV rectal microbicides [6, 13]. Such products are intended to be administered in the rectum in order to prevent early viral transmission steps and thus avoid infection. Another area of great interest and advances relates with the amelioration of inflammatory bowel disease (IBD) affecting the distal colon and rectum, namely ulcerative colitis (UC) [14]. However, rectal drug administration still relies mainly on "traditional" dosage forms such as enemas, ointments and suppositories. Such vehicles, although practical, typically feature limited ability to control drug delivery past the release phase, as well as to determine local drug distribution and residence. Nanotechnology-based approaches have been shown potentially useful in circumventing parallel shortcomings of "traditional" pharmaceutical formulation approaches used for other drug delivery routes, namely oral and parenteral ones [15, 16]. The utilization of nanomedicine principles for advancing rectal drug delivery – either for treatment, diagnostics or preventive purposes – is just now starting to rise but with promising results. In this work, we revise the basics of rectal drug delivery

[1] This section has been partially submitted for publication as "Melo M., Nunes R., Sarmento B., das Neves J., Rectal administration of nanosystems: from drug delivery for diagnosis".

and explore the use of nanocarriers for this last purpose. Particular emphasis is placed on the ability of nanosystems to distribute and retain along the colorectum following rectal administration, as well as to affect local and systemic pharmacokinetics (PK).

2.1. The rectal route in medical practice: a brief overview

2.1.1. Considerations on colorectal anatomy, histology and physiology

The rectum constitutes the terminal 10-15 cm portion of the gastrointestinal tract, stretching from the rectosigmoid junction to the anal canal. This region can further be divided into three segments, namely the upper, the middle and the lower rectum [17]. Although medication is administered in the rectum, the continuum of the gastrointestinal tract determines that drugs can potentially reach much farther upstream and well into the sigmoid and descending colon. A layer of mucus (pH around neutrality and poor buffer capacity) covers the mucosa and provides protection against trauma, namely related with the natural function of the rectum, *i.e.* storage of feces before defecation [18]. This intricate network of mucin fibers (up to 5%) may also constitute a relevant barrier for drug transport along the colorectum and/or towards underlying tissues [19, 20]. Still concerning the rectal lumen, enzymatic activity is fairly low particularly when compared to the upper gastrointestinal tract [21]. Rectal microbiota display high diversity and variability although anaerobic bacteria from the *Prevotella*, *Bacteroides* and *Faecalibacterium* genera seem to prevail [22, 23]. Local microorganisms possess the potential to metabolize active molecules and contribute to their pre-systemic elimination. Hydrolytic and reductive pathways seem to be the main contributors for drug metabolism at the rectal lumen [24, 25].

The colorectal mucosa is lined by simple columnar epithelium comprising straight tubular glands extending across the thickness of the tissue, and consisting mainly of goblet cells. The limited anatomical area and the absence of villi makes the surface area available for drug permeation quite smaller than at the small intestine [26]. Enterocytes are scarce and Paneth cells are usually absent. Single lymphoid nodules and numerous immune cells lie in the underlying lamina propria, alongside blood and lymphatic vessels.

Proximal to the pectinate line where the anal canal begins, intestinal glands become rare and disappear whereas the simple columnar epithelium makes an abrupt transition to non-keratinized stratified squamous epithelium, which then becomes keratinized at the skin [27]. Blood supply and drainage of the rectum is essentially provided by rectal arteries and veins, respectively. However, blood coming from the rectum may end up reaching either the portal system (upper rectum and colon) or the inferior vena cava (middle and lower rectum) [28]. In this last case, systemic distribution of drugs bypasses the hepatic first-pass effect, which could be of value in preventing extensive premature metabolism of active compounds. Although mixed drainage is virtually unavoidable, partial avoidance of the hepatic first-pass effect has been long demonstrated [29]. Lymphatic drainage may also play a relevant part in systemic absorption (also avoids hepatic first-pass effect), namely of highly lipophilic drugs. In this case, drainage is provided by a rich network of lymphatic plexuses converging into an extramural system of lymph channels and nodes [30].

2.1.2. Local and systemic effect

Different drugs may be administered in the rectum namely by using "traditional" dosage forms such as suppositories, soft capsules, semisolids (*e.g.*, creams, gels) and enemas (**Table 1**). Although the purpose of such administration is well defined as either intending to achieve local or systemic effects, drug bioavailability will be mainly dependent on factors such as inherent physicochemical properties (*e.g.*, solubility), permeability/absorption phenomena, and administered dose. Local retention of drugs immediately following administration is essential in order to maximize pharmacological effect. While maintenance of high drug levels at the colorectum is seen as beneficial in terms of local action, it also contributes to enhanced absorption to the systemic circulation. Extensive drug spreading throughout the colon may also be required when large areas of the mucosa are affected by a pathological condition, such as it is typical in UC [31]. However, high retention and distribution of drugs at the distal colon may not be easily achievable due to continuous bowel movements and natural anatomical features that force expulsion to the exterior. Solid and semisolid dosage forms are often used to maximize retention but usually limit retrograde spreading of drugs along the colorectum;

in contrast, liquid and aerosol dosage forms allow wide drug distribution (up to the end of the transverse colon) but may feature relatively poor residence [32, 33].

Table 1: Selected examples of drugs administered by the rectal route in clinical practice, either intended for local or systemic action.

Drugs	Therapeutic indications	Dosage forms[a]
Local action		
Bisacodyl	Relief of constipation	Suppository, enema
Budesonide	Anti-inflammatory in UC	Enema, foam
Cinchocaine hydrochloride/hydrocortisone	Treatment of hemorrhoids and anal pruritus	Suppository
Glycerin	Relief of constipation	Enema, suppository
Hydrocortisone acetate	Anti-inflammatory in UC and other conditions	Foam, enema
Lidocaine hydrochloride/zinc oxide	Treatment of hemorrhoids and anal pruritus	Cream, ointment, suppository
Mesalazine	Anti-inflammatory in UC	Enema, foam, suppository
Nitroglycerin	Chronic anal fissure	Ointment
Sulfasalazine	Anti-inflammatory in UC	Enema, suppository
Systemic action		
Acetaminophen	Analgesic, antipyretic	Suppository
Artesunate	Treatment of malaria	Soft capsule
Diazepam	Epileptic and febrile convulsions, particularly in children	Solution
Diclofenac	Anti-inflammatory, analgesic, antipyretic	Suppository
Indomethacin	Anti-inflammatory, analgesic, antipyretic	Suppository

[a] Not an exhaustive list of all available dosage forms.

Transport from the colorectal lumen towards the mucosa may be limited by the ability of drugs to dissolve and diffuse across mucus. This natural fluid may play a particularly relevant barrier to the diffusion and, ultimately, to the absorption of large and poorly soluble molecules or drug delivery systems [34, 35]. Once reaching the mucosa,

drugs may further penetrate the mucosa and particularly cross the epithelial lining, thus reaching the underlying blood vessels that can allow access to systemic circulation. In general, permeability across the epithelium can be achieved via paracellular or transcellular pathways, and is greatly dependent on the intrinsic physicochemical characteristics of drugs [36].

2.1.3. Non-medicated rectal products

Several products featuring no pharmacologically active ingredients are frequently used for rectal administration. These typically include liquid or semi-solid preparations intended for lubrication and/or cleansing of the colorectum in order to aid medical procedures, management of constipation, or preparation for sexual intercourse [37-39]. The importance and knowledge on lubricant/cleansing products accrues from their recurrent use, which can potentially assist in designing novel drug products. For example, various studies have focused particularly on the safety of lubricants used during anal intercourse, providing valuable information concerning the selection of biocompatible excipients, pH and osmolality values for rectal products [40-43]. In particular, hyperosmotic products containing relatively high amounts of humectants (*e.g.*, propylene glycol or glycerol) and presenting pH values diverting from neutrality have been found able to induce epithelial damage. These data have been pivotal in the issuing of an advisory note from the World Health Organization (WHO) and other partners for the procurement of safer lubricants for sex-related use [44]. Parallel safety issues may also emerge when considering cleansing products [45, 46]. Studies involving non-medicated rectal products have further been shown useful in assessing preferences and acceptability by end-users [47].

2.2. How can nanotechnology-based approaches potentially benefit rectal drug delivery?

Nanotechnology is often seen with overwhelming optimism when considering healthcare applications, despite many well-known limitations and challenges [48]. Beyond the common hipness and popularity associated with nanomedicine, there are

actually several possible advantages of using nanocarriers that could be interesting for rectal administration of active compounds. Alongside data obtained from studies specifically addressing rectal administration of nanosystems, a set of possibilities can also be inferred from studies involving other drug delivery routes, namely the oral one. From a general perspective of pharmaceutical formulation, nanotechnology-based approaches have been successful in solving poor drug solubility, which is known to undermine the use of various promising compounds [49]. In the particular case of the rectal compartment, the low amount of fluids that are naturally present or that can be administered makes solubility a main topic to be considered. Relatively simple solutions such as nanosizing insoluble drugs may be useful. As an example, Rachmawati *et al.* [50] observed enhanced anti-inflammatory effects for D-α-tocopherol polyethylene glycol (PEG) 1000 succinate (TPGS)-stabilized curcumin nanosuspension (mean particle diameter of ≈210 nm) after multiple rectal administrations to a trinitrobenzene sulfonic acid (TNBS)-induced colitis rat model, as compared to a curcumin suspension (>7 μm and also containing TPGS). The use of drug nanocarriers can further be helpful in allowing the production of physicochemical stable systems containing labile compounds such as nucleic acids or proteins. Hard to formulate drugs may found in nanotechnology a suitable way to abbreviate degradation during storage and even upon administration in the lower gastrointestinal tract (GIT) [51]. Control of drug release is also possible with nanocarriers [52], even though other type of larger systems may be better suited for such purpose.

The distribution and retention of drugs upon rectal administration are an important factors in defining drug efficacy. Depending on specific colloidal properties, nanocarriers can help enhancing drug transport along and across mucus and coverage of the mucosal surface, as well as increase residence. This particular topic has seen important advances over recent years and is discussed in more detail over the next section. Nanosystems present the additional potential for targeted drug delivery along the colorectum, particularly when diseased tissues can be distinguished from healthy counterparts (*e.g.*, in UC or cancer) [53-55]. In these cases, distinct pathophysiological features of the mucosal wall can be used for engineering targeted nanosystems. For example, Lamprecht *et al.* [56] reported on the accumulation of negatively-charged polystyrene particles at

inflamed tissue areas of the colon following deep intrarectal administration to rats presenting TNBS-induced colitis. Such effect could be moderately enhanced by simply reducing the size of the particles from ≈1 μm or above to ≈100 nm. A clear mechanistic explanation for these observations was not provided but differences in bioadhesion as related to different properties of local mucus (thicker but possessing looser structure in colitis) and tissue charge (more positive in colitis [57]), as well as disruption of the epithelial barrier, may be involved. Indeed, differences between differently sized negatively-charged particles were not seen when considering normal colon mucosae, including in healthy rats [56]. The presence of an abnormal number of immune cells in inflammatory conditions of the colon may also have contributed to the enhanced uptake of NPs and, thus, tentatively lead to increased drug concentrations at damaged tissues [58]. Importantly, a first in-human trial testing either PLGA-based NPs (≈250 nm) or microparticles (≈3 μm) failed to essentially replicate previous animal data [59]. Particles were administered intrarectally in a normal saline enema containing 10% albumin, which was retained for 2 h *in loco* before expulsion, and further probed by confocal laser endomicroscopy. While larger particles, but not NPs, were shown to mildly target ulcerated tissue in IBD patients, accumulation was overall much lower. Although a more objective explanation for these discrepancies seems elusive, interspecies variability and substantial differences in the colloidal properties of particles (polymer type, size, surface charge, albumin coating) may be accountable. Again, proper engineering of nanosystems appears to be of paramount importance for overall performance.

Targeting specific biological moieties that are naturally present at the epithelium by using functionalized nanocarriers may further be useful as a strategy for enhancing drug delivery, namely for cell targeting [60]. Another interesting feature of nanosystems has to deal with the possibility of modulating drug permeability at the colon and, thus, influence bioavailability. Nanoparticulate systems are able to actually penetrate colorectal tissue (**Figure 1**), which could be beneficial for allowing the establishment of a drug depot that sustains local drug residence, as well as for targeting structures or cells present at the lamina propria [61]. Data from experiments using *ex vivo* human rectal mucosa biopsies obtained from healthy individuals and IBD patients indicate that translocation of negatively-charged PLGA-based NPs (≈250 nm) across intact epithelium is limited but

increases in damaged mucosa [59]. Limited evidence further suggests that at least some type of nanosystems, namely liposomes of ≈300 nm in diameter, may partially reach systemic circulation upon rectal delivery and distribute throughout various distant tissues [62]. However, recent work using PEG-modified silica-based nanocapsules (≈120 nm) indicates that such absorption and long distance recovery of NPs is typically scanty following intrarectal administration [63]. Lack of systemic exposure to nanosystems may be considered of interest as it may favors localized pharmacological action and reduced potential toxicity.

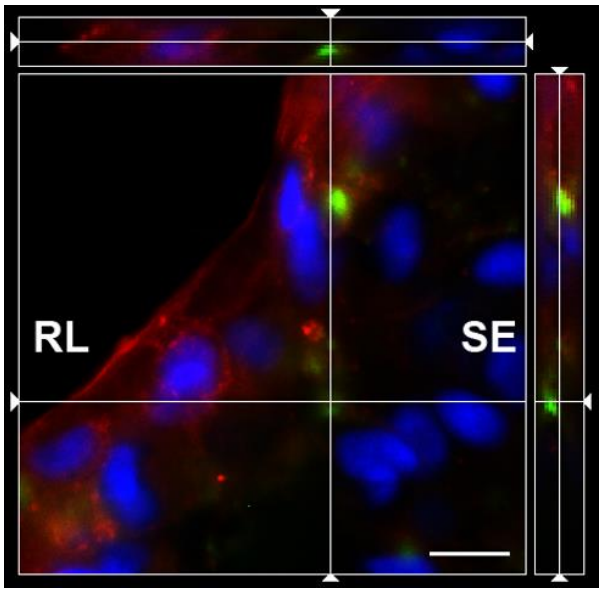

Figure 1: Evidence of the penetration of nanoparticles in pig rectal mucosa. Confocal microscopy images after 2 h *ex vivo* incubation with poly(ethylene oxide)-modified, rhodamine-123-labeled poly(ε-caprolactone) nanoparticles (≈200 nm mean diameter). Green, blue, and red signals are from rhodamine-123-labeled poly(ε-caprolactone) (nanoparticles), Hoechst 33342 (DNA), and wheat germ agglutinin, Alexa Fluor 594 conjugate (sialic acid/Nacetylglucosaminyl residues at cell membranes/mucin), respectively. Scale bar = 10 μm and z-axis range = 16 μm. RL and SE stand for rectal lumen and subepithelium, respectively. Adapted with permission from [61]. Copyright (2013) American Chemical Society.

Lastly, a note is due to nanosystems possessing intrinsic pharmacological activity rather than acting as drug carriers. Dendrimers, in particular, have been comprehensively tested for their potential use as rectal microbicides in order to prevent infection by multiple pathogens, including HIV [64-66]. The colloidal nature of these structures allows enhancing exposure of surface chemical groups responsible for interactions with pathogen or host molecular targets involved in transmission. Silver NPs [67, 68] and glass beads coated with nanolayers of silver [69] have also been studied for the treatment of colitis due to their intrinsic anti-inflammatory activity.

2.3. Distribution of nanosystems in the colorectum following rectal administration

One of the potential advantages of using nanosystems has to deal with their ability to modulate self-distribution, as well as residence, along the terminal GIT. Transport in the colorectal lumen and beyond is conditioned by several factors related not only with the mucosal environment but also with the intrinsic characteristics of nanosystems such as size and surface chemistry. Indeed, as discussed in the following, suitable engineering of the colloidal features of nanosystems plays a key role in modulating biodistribution upon rectal administration.

As in the case of other type of materials, nanosystems typically undergo rapid depletion towards the exterior following administration in the rectum as a result of the natural bowel movement and defecation reflex. The presence of stool or practice of anal sex may additionally influence clearance. Moreover, retrograde transport is thought to be naturally limited, although different studies demonstrated that nanosystems can indeed reach far into the upper parts of the colon and feature more or less extended residence. For example, Zavaleta *et al.* [63] showed that surface-enhanced Raman scattering (SERS) NPs (≈120 nm) – comprising gold core/silica shelled capsules modified at the surface with PEG (2-5 kDa) and 64Cu – distributed throughout the colon following rectal administration to mice, as assessed by Raman imaging and micropositron emission tomography. Although the depth of catheter insertion into the colon was not disclosed (but likely several centimeters) nor quantitative colon spreading studies were performed, data suggest that NPs could be transported retrogradely, at least in some animals (**Figure 2**) [63]. The use of a relatively large volume (200 µL) may aided spreading. Also, distribution into the blood and other organs (including the spleen and liver) was shown negligible. Another report by the same group confirmed these data and further indicated that NPs were rapidly washed out from the colon following a single rectal administration, without apparent signs of systemic toxicity [70].

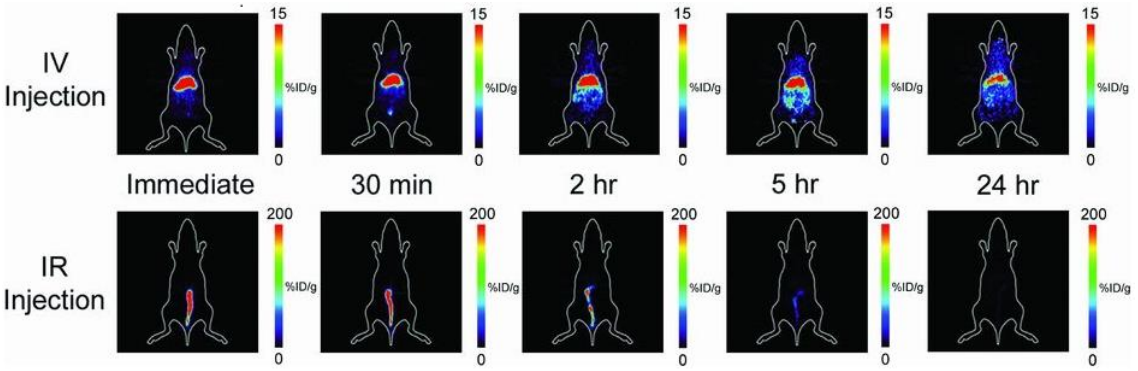

Figure 2: Micropositron emission tomography images of the accumulation of 64Cu-modified SERS nanoparticles post intravenous (IV) injection (top panel) versus post intrarectal (IR) injection (bottom panel). The images represent a coronal slice of a single mouse taken at various time points; immediately, 30 min, 2 h, 5 h, and 24 h after either IV or IR injection. Notice the significant difference in the accumulation of 64Cu-modified SERS nanoparticles in mice receiving an IV injection, where uptake is localized to the liver, versus mice receiving an IR injection, where uptake is localized to the colon. Colored scale bar to the right of each image represents 64Cu-SERS uptake where red represents most uptake and black represents no uptake in units of percentage of injected dose per gram (%ID g^{-1}). Reprinted with permission from [63]. Copyright (2011) Wiley-VCH Verlag.

One particularly limiting physiological factor for colorectal distribution and retention is the presence of mucus, a viscoelastic gel composed of water and entangled mucin fibers, which constitutes a stringent barrier for particle diffusion [71, 72]. The ability of nanosystems to bond mucins determines their adhesive behavior and, thus, overall mobility. The use of mucoadhesive nanosystems has been conventionally considered beneficial for enhancing drug retention at mucosal sites and a popular approach to improve either local or systemic drug bioavailability [73]. Strategies involving the modification of the surface of nanosystems in order to yield enhanced molecular entanglement and covalent or non-covalent bonding with mucin fibers are recognized for increasing mucoadhesiveness [74]. However, strong adhesion of nanosystems to mucins impairs effective transport across mucus thus limiting distribution not only towards the underlying mucosal tissue but also retrogradely into the colon. Also, mucoadhesive systems are mostly immobilized at the outer, non-adherent layer of mucus, which undergoes continuous and rapid clearance [71, 75]. Conversely, mucus-diffusive (or mucus-penetrating) nanosystems can migrate to the inner layer of mucus, which is less prone to luminal shearing, and even have easier access to underlying tissues. Therefore, the use of non-mucoadhesive nanosystems has been gaining advocates over recent years as an interesting approach to promote not only spreading along mucosal sites but also enhance overall residence [76, 77]. The most successful strategy for producing mucus-diffusive nanosystems has been developed by Hanes and collaborators [78-80] and involves dense surface modification of nanosystems with PEG featuring adequate molecular mass for preventing interactions with mucin, alongside adequate particle size

control down to dimensions that allow fitting the mucus mesh spaces. Still, other approaches have also been recently proposed including surface modification of nanosystems with alternative non-mucoadhesive polymers or association with mucolytic agents [81-84]. In the specific case of rectal delivery applications, Maisel *et al.* [8] modified polystyrene NPs of different sizes with PEG (5 kDa) and found that this approach led to an overall increase in migration of nanosystems across mucus, as well as to an extensive coverage of the epithelial surface after administration to mice (**Figure 3**). Contrariwise, mucoadhesive counterparts (*i.e.*, without PEG modification) distributed poorly and largely aggregated at the intestinal lumen. Size of NPs also played an important role: 40-100 nm PEG-modified NPs featured a more uniform distribution along the epithelial surface as compared to larger ones (200-500 nm). This fact may well be associated to the available space within the colorectal mucus mesh, which sterically hinders transport of larger nanosystems. Another important finding of this study relates with different patterns of NP transport due to pathological changes. For example, colorectal distribution of 200 nm mucus-diffusive NPs was enhanced in a TNBS-induced colitis mouse model, which feature a thicker yet looser mucus barrier, as compared to healthy animals. Moreover, mucus-diffusive NPs appeared to accumulate better than mucoadhesive counterparts in ulcerative lesions [8]. The use of a hypotonic vehicle was further shown beneficial for administering mucus-diffusive NPs due to the promotion of convective transport [8, 85]. In a subsequent study by the same research group, similar behavior was also described for NPs modified with PEG of higher molecular mass (40 kDa) after intrarectal administration to mice [86]. Despite these interesting findings, actual time-dependent, quantitative retrograde migration and overall colon retention of PEG-modified NPs were not determined. Assessment of such data is paramount for fully understanding the potential of mucus-diffusive NPs as carriers for rectal drug delivery and, thus, additional investigation is required.

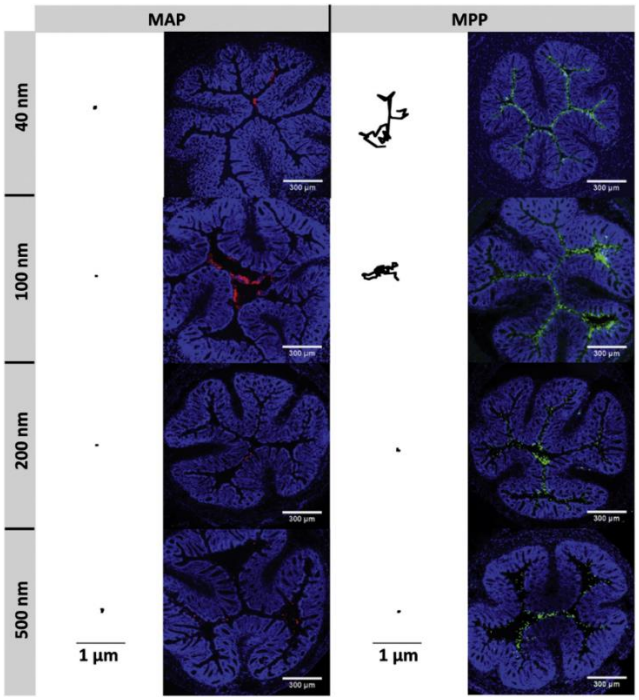

Figure 3: Trajectories in colorectal mucus and distribution of mucoadhesive particles (MAP) and mucus-penetrating particles (MPP) in the mouse colorectum. Trajectories representative of three seconds of movement for 40, 100, 200, and 500 nm MAP and MPP in mucus on freshly excised mouse colorectal tissue as assessed by multiple particle tracking video microscopy. Black scale bars indicate 1 µm for all trajectories. Distribution in transverse colonic cryosections after rectal administration of 40, 100, 200, and 500 nm MAP (red) or MPP (green). Cell nuclei are stained with DAPI. White scale bars indicate 300 µm for all distribution images. Images are representative of $n \geq 3$ mice. Reprinted from [8], Copyright (2014), with permission from Elsevier.

2.4. Nanosystems for rectal drug delivery and diagnosis

This section provides different examples of nanotechnology-based drug delivery or diagnostics systems intended for rectal use or, at least, holding such potential. As an important note, many of the discussed studies were performed using deep administration of nanosystems across the anal canal and into the colon of rodents, often not disclosing the distance at which instillation was performed. Although direct translation of such type of intracolonic administration may not be clinically feasible or even possible, relevance to rectal drug delivery by means of nanocarriers seems undeniable. Also, the following sub-sections have been organized according to the type of nanocarriers being used for the sake of systematization. These include polymeric nanosystems (NPs and micelles), lipid-based nanosystems (nano-liposomes, solid lipid NPs – SLN – and nanoemulsions) and protein-based NPs, as well as other nanosystems not fitting the previous categories.

Particular focus is given to *in vivo* performance aspects of developed systems such as PK, safety and efficacy.

2.4.1. Polymeric nanoparticles

NPs composed of biocompatible polymers are among the most promising drug nanocarriers currently being tested for medical use [87]. Applications in rectal drug delivery is no exception, with most examples available in the literature being based on polymers such as PLGA [88-90], poly(lactic acid) (PLA) [91], chitosan [92, 93] and methacrylic acid copolymers [94, 95]. For instance, Lamprecht and colleagues [88] proposed PLGA-based NPs for the oral and rectal delivery of tacrolimus, an immunomodulatory drug, in order to manage colitis. Drug-loaded NPs (≈107 nm) were shown effective in reducing inflammation in either TNBS-induced or oxazolone-induced colitis rat models after multiple daily intracolonic 8 cm deep instillations. Noticeable, local administration of tacrolimus-loaded NPs was superior in mitigating colitis as compared to the free form of the drug or even after oral use of drug-loaded NPs. Direct access to diseased areas of the gut (contrary to what happens upon oral administration) and preferential association to/penetration of ulcerated regions by NPs (as assessed *ex vivo*), as well as reduced local metabolism and P-glycoprotein-mediated efflux of tacrolimus, were the reasons claimed as accountable for observed therapeutic outcomes [88]. More recently, another team again led by Lamprecht attempted at further improving the association of PLGA NPs to inflamed tissues by decorating the surface of the nanosystems with either peanut or wheat germ lectins [89]. Rationale for this approach was based on the ability of such targeting moieties to bind colonic mucins. Surface functionalization of NPs led to enhanced selectivity of NPs towards inflamed tissues, particularly in the case of peanut agglutinin, as assessed in TNBS-induced or oxazolone-induced colitis murine models (**Figure 4**). These observations translated, at least partially, into improved efficacy in the management of colitis in both animal models when lectin-functionalized PLGA NPs loaded with the anti-inflammatory steroid betamethasone were administered into the colon (4 cm deep insertion), as compared to plain drug-loaded PLGA NPs [89]. A composite nanocarrier based on PLGA has also been recently proposed for the delivery of various siRNAs silencing pro-inflammatory mediators [96].

The system comprising calcium phosphate/siRNA complexes encapsulated into PLGA NPs and further coated by polyethyleneimine (≈150 nm and featuring positive zeta potential) was shown able to effectively silence tumor necrosis factor α (TNFα), IP-10 and KC *in vivo*, thus leading to a reduction in inflammatory symptoms in a dextran sulfate sodium (DSS)-induced murine model of colitis. These results were correlated with the ability of NPs to effectively distribute throughout the distal colon following rectal administration and deliver siRNA to the cytoplasm of epithelial and immune cells not only present at the mucosa but also at mesenteric lymph nodes [96].

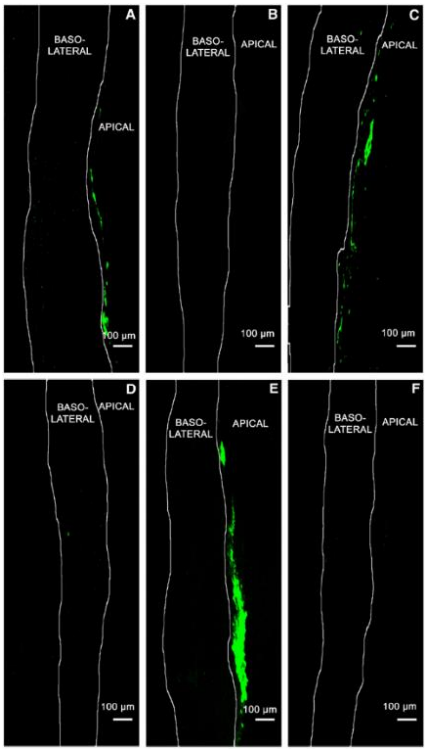

Figure 4: Confocal laser scanning microscopy bioadhesion images were recorded from cryomicrotome cross-sections of the colonic tissue 24 h after rectal administration of surrogate lectin-conjugated NPs (fluorescently labeled polystyrene NPs) and unconjugated NPs: (A, B) polystyrene-based NPs, (C, D) wheat germ agglutinin-modified, polystyrene-based NPs, and (E, F) peanut agglutinin-modified, polystyrene-based NPs. The accumulation of particles in inflamed tissue increased in the order (A) unconjugated NPs < (C) wheat germ agglutinin-modified NPs < (E) peanut agglutinin-modified NPs. (B, D, F) Low fluorescent signals obtained from the respective healthy groups underlined the selectivity towards inflamed tissues. Reprinted from [89], Copyright (2014), with permission from Elsevier.

Chitosan and derivatives have been another interesting choice of materials for producing NPs intended for rectal drug delivery, namely of biopharmaceuticals [92, 93]. In an interesting report, Zuo *et al.* [92] proposed NPs of galactosylated low molecular weight chitosan (Gal-LMWC) complexing with antisense oligonucleotide (ASO) against TNFα to be potentially used in the management of IBD. Main intended target cells for

the ASO were macrophages due to their role in TNFα production in experimental colitis, as well as to their ability to express a galactose/*N*-acetyl galactosamine-specific lectin, which was used as a natural target for galactose residues in Gal-LMWC NPs. Macrophage targeting and ASO accumulation were demonstrated both *in vitro* and *in vivo* at the colon following administration of Gal-LMWC/ASO NPs to mice featuring TNBS-induced colitis (**Figure 5**). Moreover, favorable therapeutic outcomes were observed following single or repeated 4 cm deep intracolonic treatment of TNBS-induced or $CD4^+$ $CD45RB^{hi}$ T cell-mediated colitis murine models, respectively [92].

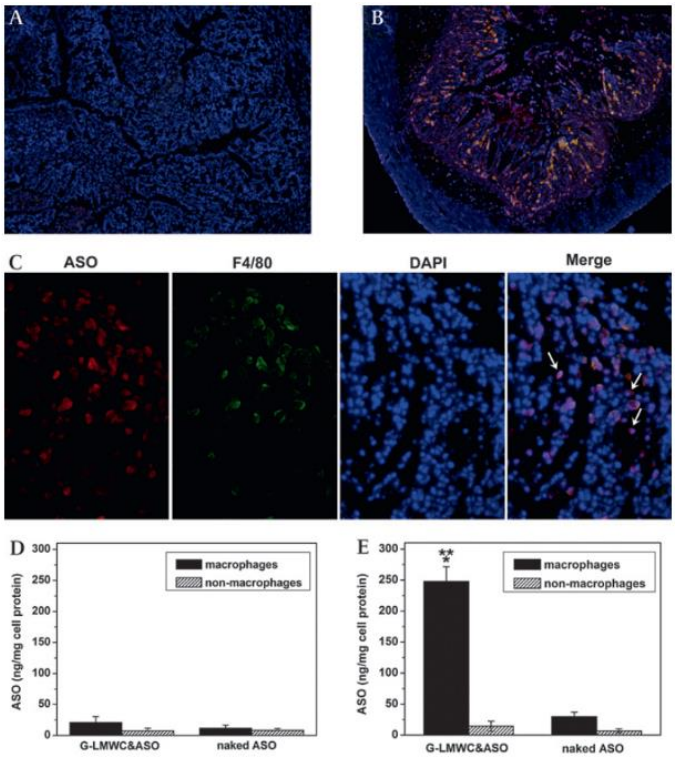

Figure 5: Gal-LMWC (galactosylated low molecular weight chitosan) effectively delivered antisense oligonucleotide (ASO) into activated macrophages in mice with colitis. Gal-LMWC/HEX (6'-carboxy-2',4,4',5',7,7'-hexachlorofluorescein-labelled)-ASO complex was injected into normal mice or TNBS-treated mice by intracolonic administration at a dose of 5 mg ASO/kg body weight. Frozen sections of the colons were stained with F4/80 antibody. Results are from (A) control mice (magnification ≈100) and (B) mice with TNBS-induced colitis (magnification ≈100), 24 h after ASO injection. (C) Arrows indicate the accumulation of HEX-ASO in colonic activated macrophages (magnification ≈400). Red: ASO; green: F4/80; blue: 49,6-diamidino-2-phenylindole (DAPI) nuclear staining. Colonic macrophages were isolated from normal or inflamed colon 24 h after the intracolonic administration of Gal-LMWC/ASO complex or naked ASO by fluorescence-activated cell sorting. The other cells during the separation process were collected as 'non-macrophages'. Quantification of cellular uptake of ASO in (D) control mice and (E) TNBS-treated mice was determined by Southern blotting. n=5-6 mice per group; values are expressed as the mean ± standard error of the mean. (*) denotes $p<0.05$ compared with macrophages from mice treated with naked ASO; (**) denotes $p<0.05$ compared with non-macrophages from Gal-LMWC/ASO nano-complex-treated mice. Reproduced from [92], Copyright (2010), with permission from BMJ Publishing Group Ltd.

The commercially available Eudragit® excipient series comprises methacrylic acid copolymers featuring several interesting properties for supporting drug delivery at the colon [97]. The versatility of these materials has been explored for producing drug nanocarriers for rectal administration. For example, Lamprecht and collaborators [94] proposed clodronate as an alternative drug for IBD due to its suppressive effects on macrophage activity and used Eudragit® RL-based NPs as carrier system. The positively charged ammonioalkyl methacrylate copolymer allowed high association efficiency of negatively charged clodronate (≈90%), yielding nanosystems around 120 nm in diameter and featuring ensemble positive surface charge. Additional rationale for using such NPs was their presumed ability to strongly interact with negatively charged mucin, which could restrict unhindered access to the underlying mucosa, as well as macrophages therein, only at areas of inflammation and with limited mucus protection. Although mitigation of inflammation *in vivo* using both TNBS-induced or oxazolone-induced colitis mouse models was mild, multiple treatment with drug-loaded Eudragit® RL-based NPs was superior to that with clodronate in solution [94]. The same research group also recently explored the same rationale for enoxaparin by producing and testing similar nanosystems based on either Eudragit® RL or Eudragit® RS [95]. Parallel therapeutic findings were reported when using NPs in a TNBS-induced colitis murine model, thus supporting, at least in part, the usefulness of positively charged NPs in pathologies associated with mucus disruption.

Apart from therapeutic purposes, a report by Cheng *et al.* [98] demonstrated that gadopentetic acid-loaded chitosan NPs (≈420 nm) were able to increase the intensity of magnetic resonance imaging signal of the colon following rectal administration to rats. Presented data suggest that this novel contrast agent may be a promising tool for the diagnosis of bowel disease, although more specific work needs to be conducted (*e.g.*, in colonic disease models) [98]. Future challenges for developing polymeric NPs for diagnostics are related with low specificity, sensitivity and probe stability of used agents. Functionalizing nanosystems with targeting moieties may provide an interesting way to circumvent these problems. In this sense, Sakuma and colleagues [99-101] proposed polystyrene NPs loaded with the fluorescent label coumarin-6 and coated with peanut agglutinin (targeting agent), poly(methacrylic acid) (linker for the agglutinin) and poly(*N*-

vinyl acetamide) (reduces non-specific binding to the mucosa) for early detection of the Thomsen-Friedenreich (TF) antigen. This last disaccharide is overexpressed in colorectal cancer and constitutes a well-established biomarker for early diagnosis. Developed nanosystems (≈250-400 nm) were deeply administered into the colon of mice bearing colorectal cancer by using a spray catheter, and were shown helpful in aiding the detection of cancer lesions by fluorescence colonoscopy [102]. Although apparently specific and sensitive, wide colonic spreading of the developed nano-probes following more clinically relevant rectal administration practices was not demonstrated, which could undermine usefulness for colonoscopic analysis of the upper regions of the colon. However, probe and administration technique seem feasible for detecting lesions at the colorectum [103]. Additionally, results from a study in rats further supported that these nano-probes were safe after a single rectal administration at a 50 mg/kg dose [104].

2.4.2. Polymeric micelles

Micelles, particularly those of polymeric nature, hold great potential and popularity as drug delivery systems for mucosal use. Typically smaller than 100 nm and composed of various amphiphilic copolymers, such systems are able to incorporate poorly water soluble compounds into the hydrophobic core while maintaining high colloidal stability owing to the hydrophilic surface [105]. A few studies demonstrating the potential of micelles for rectal drug delivery have been published over recent years. For example, Seo *et al*. [106] described micelles (≈13 nm) composed of polysorbate 80 and poloxamers as carriers for docetaxel. Aqueous dispersions of micelles featured both thermosensitive and mucoadhesive properties, which could benefit residence upon rectal administration. When administered intrarectally (4 cm deep) to rats, drug-loaded micelles were able to improve the bioavailability of docetaxel by roughly 10-fold as compared to oral Taxotere®, the commercial intravenous formulation of docetaxel. Moreover, micelles were show safe after histological analysis, and presented improved or comparable efficacy to oral or intravenous treatment with Taxotere®, respectively, when tested in nude mice featuring subcutaneous KB cell-derived tumors (**Figure 6**) [106]. In a subsequent work, the same research group attempted to further optimize the delivery system by incorporating sodium taurocholate, a permeability enhancer [107]. However,

differences in bioavailability for micelles with and without sodium taurocholate (≈15 nm) were, at best, mild and *in vivo* efficacy was unaffected. In a recent study, Courthion *et al.* [108] proposed micelles as carriers for cyclosporine A, an immunosuppressive drug, for localized therapy of UC. Drug-loaded nanosystems were produced using methoxy PEG-hexyl substituted PLGA and featured mean diameter of 40-60 nm and near neutral zeta potential as conferred by the dense self-assembled PEG corona. When tested in a TNBS-induced colitis murine model after 4 cm deep intracolonic instillation, micelles were able to provide increased drug levels at the colon as compared to those obtained in healthy animals, which was correlated with the higher ability of nanocarriers to interact with inflamed tissues. Furthermore, drug-loaded micelles were as effective as standard intrarectal 5-aminosalicylic acid treatment in managing colitis, thus demonstration the potential therapeutic usefulness of these systems [108].

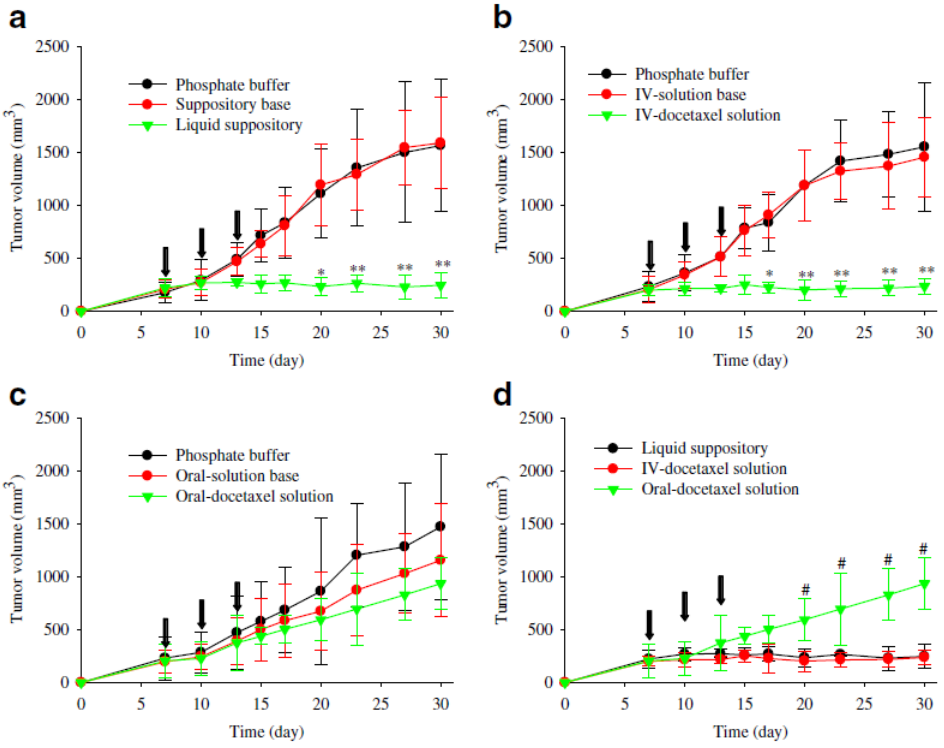

Figure 6: Antitumor efficacy of (a) rectally administered docetaxel-loaded micelles (referred to as 'liquid suppository'), (b) intravenously administered docetaxel solution, and (c) orally administered docetaxel solution to tumor-bearing nude mice on days 7, 10 and 13 (noted by black arrows). The last graph (d) represents the comparative antitumor effects of all three formulations. (*) Significantly smaller than phosphate buffered saline (PBS) and drug-free micelles (referred to as 'suppository base'; $p<0.05$). (**) Significantly smaller than PBS and drug-free micelles (referred to as 'suppository base'; $p<0.05$). (#) Significantly larger than docetaxel-loaded micelles (referred to as 'liquid suppository') and intravenous docetaxel solution ($p<0.05$). Each values represents the mean ± standard deviation ($n=5$). Reprinted from [106] with permission of Springer Science+Business Media, Copyright (2013).

2.4.3. Nano-liposomes

Liposomes are highly versatile nanocarriers that, depending on specific composition and processing, may feature dimensions in the range of several tens to hundreds of nanometers. Their spherical but fluid structure formed by a variable number of concentric phospholipid-based bilayers surrounding an aqueous core allows the convenient incorporation of both hydrophilic and hydrophobic drugs [109]. Moreover, liposomes are typically biocompatible and easily engineered in order to allow, among other possibilities, enhancing interactions with the surrounding environment or targeting drugs to cellular and sub-cellular levels [110]. A few studies on the use of nanosized liposomes for rectal drug delivery have been described. Commonly used liposome-based commercial transfection reagents such as Lipofectamine® 2000 have been tested for intrarectal administration of siRNA against TNFα in the context of UC, often with interesting results [111, 112]. Although being useful and convenient solutions for establishing proof-of-concept, translation of commercial liposomal reagents towards clinical use may be hampered due to toxicity and regulatory issues, among other reasons. Also, lipoplexes described in the literature are often poorly characterized regarding colloidal properties, including the effect of such properties on delivery outcomes. Thus, liposomal formulations that are more suitably engineered for potential medical use may be preferable. For example, Jubeh *et al.* [113] proposed negatively-charged nano-liposomes as carriers for catalase, superoxide dismutase or tempamine (a synthetic antioxidant) for treating UC. Different formulations (≈390-420 nm) were introduced deeply into the colon of rats featuring dinitrobenzene sulfonic acid (DNBS)-induced colitis. Treatments were performed every 12 h for 3 days and were shown effective for superoxide dismutase and tempamine (as well as their combination) in reducing colonic inflammation. Authors advocated that enhanced effects observed for liposomal formulations as compared to free active agents were related to increased uptake of nanosystems by the injured mucosa although no PK data has been provided in order to support such assumptions [113].

Modified nano-liposomes were recently proposed for mucosal immunization upon rectal administration, namely against hepatitis B virus infection [114]. The nanocarrier

comprised a 1,2-dipalmitoyl-sn-glycero-3-phosphocholine bilayer engulfing a solid fat core (mainly glyceryl tripalmitate), and was loaded with hepatitis B surface antigen and monophosphoryl lipid A (adjuvant). This hybrid liposomal system (≈210 nm) was able to maintain structural stability and immunogenicity of the antigen and, when administered deeply (6-7 cm) into the colon of rats, conferred an increased mucosal immune response as compared to a commercial intramuscular vaccine, GeneVac-B™. According to the authors of the study, these results seem to reinforce both the importance of direct antigen exposure to lymphoid tissue at the colon and the use of proposed nanocarriers in eliciting strong mucosal response [114].

Beyond local gut therapies or immunization, Moawad *et al.* [115] studied the possibility of using liposomes for the systemic administration of tizanidine, a muscle relaxant, by the rectal route. Optimized drug-loaded nano-liposomes (≈150 nm) based on phosphatidylcholine, cholesterol and polysorbate 80 were incorporated into a thermosensitive gel for enhanced retention before rectal delivery to rabbits. Bioavailability of tizanidine was increased by roughly 2.2-fold and 1.6-fold as compared to oral delivery of tizanidine in aqueous solution and rectal delivery of the free drug in the same thermosensitive gel, respectively. The authors of the work argued that partial avoidance of the hepatic first-pass effect and the permeation enhancement provided by drug incorporation into nano-liposomes may explain these meager differences [115].

2.4.4. Solid lipid nanoparticles

SLN are popular nanocarriers typically composed of one or more lipids and having a long and well-established track record in the drug delivery field [116]. A few examples of their potential application to rectal delivery have been described in the literature for several years but typically of preliminary nature, failing to demonstrate clear advantage over conventional formulation approaches, or using indirect delivery (*e.g.*, using surgical colorectal loops) [117-119]. For instance, Din *et al.* [120] recently developed an interesting double reversed thermosensitive dosage form comprising ≈190 nm flurbiprofen-loaded SNL (solid at room temperature but melting at just under body temperature) incorporated into poloxamer-based gels presenting opposite thermal

behavior. When deeply administered (4 cm) into the colon of rats, the formulation was apparently safe and able to increase bioavailability by around 8-fold as compared to flurbiprofen in solution. However, only mild differences were reported for the thermosensitive gel containing a dispersion of the free drug, thus suggesting that the value of SLN was reduced as compared to the ability of the overall liquid system to undergo sol-gel transition upon administration and promote prolonged retention at the colon [120]. More recently, the same group proposed a similar system for the rectal administration of the anticancer drug irinotecan but, again, no clear differences were observed between thermosensitive gels containing drug-loaded SLN or the free drug [121]. Both systems provided similar plasmatic levels of irinotecan in rats, and only mild differences in antitumor efficacy favoring SLN-in-gel when tested in a xenograft murine model bearing subcutaneous squamous cell tumors.

2.4.5. Nanoemulsions

Nanoemulsions are liquid systems featuring two immiscible phases (typically oil-in-water) and are distinguished from "regular" emulsions due to the submicron diameter of the droplets of the internal phase [122]. These systems have been shown particularly suitable for the delivery of hydrophobic compounds (namely those labile to hydrolysis and oxidation), and have the potential advantage of increasing GIT retention time and distribution, which may favor enhanced bioavailability [123, 124]. Nanoemulsions have been considered for rectal administration with considerable success. For instance, Kim *et al.* [125] developed simple ethyl oleate-based nanoemulsions featuring mean droplet diameter values in the range of 40-150 nm for the delivery of indomethacin, a non-steroidal anti-inflammatory drug. Formulations were stabilized by using polysorbate 85 at different concentrations (15-40% relative to ethyl oleate content) and were intended to be used as self-emulsifying system upon mixing with physiological fluids. An optimized drug-loaded system (70% ethyl oleate/30% polysorbate 85 and containing 5.625 mg of drug) was incorporated into hollow-type gelatin/glycerin suppositories and tested in rats. Values for plasmatic maximum concentration (C_{max}) and area-under-the-curve up to 12 h ($AUC_{0\text{-}12h}$) were significantly higher than those obtained for similar suppositories containing plain indomethacin. These results were likely due to the increased solubility

of the drug after nanoemulsion formation, as well as the permeability enhancement effect conferred by the presence of surfactants [125]. In another work, Sznitowska *et al.* [117] tested a nanoemulsion (≈200 nm mean droplet diameter) for the rectal administration of diazepam as an anticonvulsant. The nanoemulsion comprised 0.4% diazepam, 20% Miglyol® 812, 1.2% egg lecithin, 2.0% poloxamer 188, 1.8% glycerol, 0.02% α-tocopherol, 0.18% methylparaben and 0.02% propylparaben. When compared to a commercially available rectal solution containing high levels of ethanol (10%) and propylene glycol (40%), the nanoemulsion allowed obtaining similar values of C_{max} and $AUC_{0\text{-}24h}$ in rabbits. Such results could be considered promising since the nanoemulsion may constitute a safer alternative according to the authors [117]. Overall, and despite these two interesting examples, additional work on rectal drug delivery using nanoemulsions has been nearly non-existing over the last decade and additional research is required.

2.4.6. Protein-based nanoparticles

Several proteins such as albumin and gelatin are among the most versatile, biocompatible and well-studied matrix-forming materials for producing drug nanocarriers [126]. Again, a few examples have been described in the literature concerning the rectal use of such systems. For instance, Lozano-Pérez *et al.* [127] proposed resveratrol-loaded silk fibroin-based NPs (≈68 nm) for managing UC. Fibroin is a biocompatible natural protein in which the repetitive amino acid sequence Gly-Ala-Gly-Ala-Gly-Ser is abundant. The promising *in vitro* immunomodulatory activity recorded for resveratrol-loaded NPs was translated into noticeable anti-inflammatory effects in a TNBS-induced colitis rat model following daily intracolonic administration for seven days, as assessed by multiple anatomical, biochemical and gene expression assays. Importantly, incorporation of resveratrol into NPs led to an improved effect over resveratrol in suspension, likely due to enhanced local bioavailability, which could have been originally impaired by the poor aqueous solubility of the drug [127]. A follow-up work by the same group also evidenced the intrinsic mild anti-inflammatory activity and mucosa protective effects of silk fibroin NPs (180 nm) both *in vitro* and *in vivo* following rectal administration to rats featuring TNBS-induced colitis [128].

Potential application of protein-based NPs in diagnostics has also been addressed. Cohen & Margel [129] developed albumin NPs loaded with the near infrared (NIR) dye IR-783. The system was additionally functionalized at the surface with peanut agglutinin, anti-CEA antibody or anti-TAG-72 antibody in order to target TF antigen, carcinoembryonic antigen (CEA) or tumor associated glycoprotein-72 (TAG-72), respectively. NPs were shown useful for detecting cancer lesions by NIR imaging in excised colon tissues from an orthotopic mouse model bearing LS174t cell-derived tumors expressing high levels of TF antigen, CEA and TAG-72 (**Figure 7**). One particular important issue concerning specificity/sensitivity of the technique was related with the proper selection of the interval between rectal administration and imaging, since an adequate amount of time was essential to allow self-washing or lavage of NPs from healthy tissue areas [129]. While it is not clear if and how such NPs could be translated into live animals/humans, this study seems to reinforce the utility of specific cell targeting at the colon as an interesting strategy for developing improved diagnostics tools.

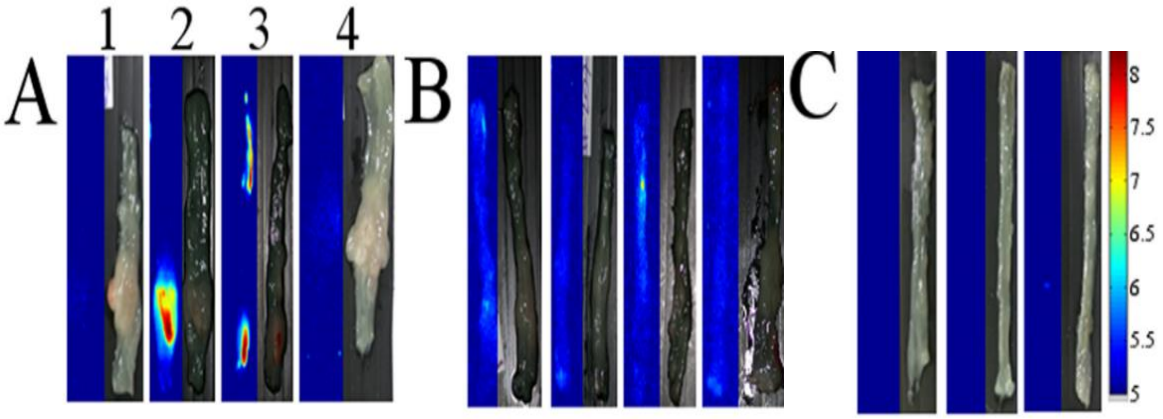

Figure 7: Logarithmically scaled fluorescent and grayscale images of typical (A) LS174t and (B) HT29 (low levels of CEA and TAG-72) colon tumor cell lines treated with (1) non-conjugated and (2) anti-CEA and (3) anti-TAG-72 conjugated NIR fluorescent albumin NPs; (4) represents untreated tumor cell lines. (C) Represents typical colons of healthy mice treated with (1) non-conjugated and (2) anti-CEA and (3) anti-TAG-72 conjugated NIR fluorescent HSA nanoparticles. 44 mice (each set of experiment was done with 4 mice) were anesthetized and treated with 0.1% particle dispersion in PBS, via the anus. Twenty minutes later the colons were extensively washed with PBS and were then allowed to recover for 4 h. The colons were then removed and treated as described in the experimental part. Images acquired at 780/800 nm with an Odyssey® Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). Reproduced from [129] under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), Copyright Cohen and Margel; licensee BioMed Central Ltd. 2012.

2.4.7. Other nanosystems

Several other nanosystems not quite fitting the sub-sections above have been further proposed for rectal administration. McCarthy *et al.* [130] studied the possibility of using siRNA/amphiphilic cationic cyclodextrin nano-complexes (≈240 nm) for TNFα

silencing in the context of IBD. The nanosystem was shown effective in maintaining siRNA stability in media mimicking basic features of colonic fluids, and able to successfully transfect activated mouse macrophages *in vitro*. When tested in a DSS-induced murine model of colitis, nano-complexes were able to significantly reduce TNFα expression at the proximal colon after intrarectal administration. However, amelioration of clinical signs was mild and only significant for colon weight, which seems to indicate that the proposed approach could be merely useful as an adjuvant to more conventional treatment of UC [130].

In another recent and interesting work, Samizadeh *et al.* [131] studied several *in vitro* and *in vivo* features of amprenavir-PEG-Bac7 nano-conjugates (no size reported) in order to attest their potential as a microbicide for preventing rectal HIV transmission. The incorporation of the cell penetrating peptide (CPP) Bac7, as well as the adequate selection of PEG molecular weight, were shown essential in allowing maintenance of the native antiretroviral activity of amprenavir. The full nano-conjugate was not tested *in vivo* but preliminary data for fluorescent labeled amprenavir-$PEG_{3.4kDa}$-FITC and Bac7-$PEG_{3.4kDa}$-FITC surrogates suggested that prolonged colorectal retention was possible due to the presence of the CPP. However, more studies with the actual drug nano-conjugate are required in order to fully assess the potential of the system [131].

Lastly, Hassanzadeh *et al.* [132] proposed carbon nanotubes/2-arachidonoylglycerol (2-AG) complexes for intrarectal administration. 2-AG, an experimental endocannabinoid with potential to be used in the management of UC, is characterized by rapid hydrolysis and aminated multi-walled carbon nanotubes were selected as carriers. When tested in a TNBS-induced colitis rat model, multiple intracolonic daily administrations of the complexes (6 mg/kg) were able to reduce the severity of the disease, as assessed by macroscopic and microscopic evaluation of colonic tissues. Different biochemical markers of inflammation in the blood and colonic tissue also confirmed amelioration of UC. Despite promising, these data require support by additional toxicity studies regarding the safety of carbon nanotubes.

2.5. Vehicles for rectal administration of nanosystems: a role for thermosensitive enemas?

One important but largely unaddressed issue concerning the rectal use of nanosystems is the establishment of adequate vehicles for administration. While more "traditional" liquid, semi-solid or solid dosage forms could be immediately considered as possibilities, our group is strongly engaged in the development of "smart" systems that could take advantage of the local environment for enhancing distribution and retention of nanosystems. Thermosensitive enemas, which are liquid at room temperature but form gels at body temperature, could provide an interesting way to provide enhanced colorectal rectal distribution (while still liquid and immediately following administration) but also retention (upon sol-gel transition). The concept is not new but its application to the rectal administration of nanosystems has not yet been assessed [133-135].

The previous systems are based in liquid solutions of thermosensitive polymers and form, invariably, hydrophilic gels. For such reason, the term "thermosensitive gel" is also often used in allusion to the final jellified form of the originally liquid system. Thermosensitive polymers are materials that respond changes in temperature by altering their microstructure and promoting alteration of physical state. This is usually characterized by a macroscopically observable liquid to gel (sol-gel) transition [136]. The incorporation of these polymers in medical/drug carrier systems may enable modulation of drug release, distribution/retention behavior or creation of physical barriers upon thermal stimulus. One of the most important examples of thermosensitive polymers used in pharmacy is the family of compounds generically dominated by poloxamers (also commonly known by the former commercial name Pluronic® from BASF, now Kolliphor®) [137]. These last are triblock copolymers comprising poly(ethylene oxide)–b–poly(propylene oxide)–b–poly(ethyleneoxide) (PEO-PPO-PEO; general formula is: $HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$) presenting sol-gel transition temperature between 15 ºC and 40 ºC at concentrations around 10-30% (*w/v*) in water. Sol-gel transition in poloxamers occurs due to the aggregation of the PPO core, thus promoting its organization in spherical shape micelles [138]. Micellization leads to the formation of a 3D structure responsible for gel formation. Precise variation of the concentration of

poloxamers allows modifying the sol-gel transition temperature to the desired values. The use of mixtures of different poloxamers or incorporation of other excipients may also impact the sol-gel transition temperature of the final formulation [137]. The possibility of obtaining sol-to-gel transition temperatures around physiological values makes poloxamers an attractive solution in drug delivery applications. Application of poloxamers have been particularly prolific in ocular [139], cutaneous [140], buccal [141] and vaginal [142] drug delivery. Among available poloxamers, poloxamer 407 (a=101; b=56; average MW of 9,840-14,600) has been one of the most widely used because of its versatile sol-gel transition behavior and capability to aid in the solubilization of many drugs [143]. Finally, poloxamers are generally regarded as safe for mucosal use, which could be a particularly advantageous feature for the development of microbicides [144].

2.6. Conclusions and remarks

The rectal route has long been considered for medical purposes but typically seen as an alternative to other more convenient ones by both clinicians and patients/users. This last fact alone may justify why advances in rectal drug delivery and diagnostics have been relatively slow and meager, namely in the development of nanotechnology-based approaches. However, as discussed along the present manuscript, an important body of work seems to support the usefulness of nanomedicines when considering the rectal route. While systemic drug delivery may be achieved following administration, applications in therapy and diagnostics of local conditions largely dominated research over the years. The wide potential of nanotechnology for the management of IBD, namely in designing advanced drug carriers [145, 146], has been one of the main driving forces in considering nanomedicines for rectal administration. Other fields that may potentially benefit tremendously from nanotechnology-based solutions require additional investment, particularly anti-HIV microbicides.

Despite all progress, various fundamental challenges remain largely unexplored and require further research. For instance, more understanding on the distribution of nanosystems is needed since many of the successful examples presented in the literature are actually achieved by using deep intracolonic administration, which is not feasible in

humans, at least for routine use. Significant retrograde spreading (and retention) of nanosystems is important for many potential medical applications but may not be easily achievable. Proper size and surface engineering may be helpful in circumventing these issues but definitive guidance is missing. Also, many of the distribution data derived from animal models of disease, namely UC, may not be completely relevant for other states since important physiological differences can lead to distinct colorectal distribution of nanosystems. Choosing appropriate vehicles for rectal administration of nanosystems is also another topic that deserves future research. Apart from influencing distribution, retention and overall bioavailability of active compounds, transforming promising nanosystems into medicines is essential for clinical translation. Another important field of research that is yet to be properly explored comprises prevention of HIV rectal transmission. The development of microbicides has bloomed over the last decade but the role of nanotechnology, if any, is yet to be defined [147]. Finally, systemic safety issues of nanosystems administered by the rectal route are expected to be limited due to predictability low systemic exposure, but special attention needs to be paid in the case of local toxic effects. Therefore, specific work needs to be conducted, namely on a case-by-case basis.

3. Materials and methods

3.1. Materials, solvents, reagents and cell lines

Carboxylic acid-terminated PLGA (Purasorb PDLG 5004A; 50:50 lactide:glycolide molar ratio; inherent viscosity midpoint of 0.4 dl/g. corresponding to approximately 44 kDa in MW or Purasorb PDLG 5002A; 50:50 lactide:glycolide molar ratio; inherent viscosity midpoint of 0.2 dl/g. corresponding to approximately 44 kDa in MW) was kindly provided by Corbion (Amsterdam, The Netherlands). Two batches of poloxamer 407 (Kolliphor® P407) were kindly provided by BASF: a first batch from their production site at Ludwigshafen, Germany (lot WPWJ584B) and a second batch from Geismar, LA, USA (lot GNA18121C). The certificates of analysis for each batch are presented in Annex 1. Sorbic acid was acquired from Fluka (Munich, Germany), glycerin from Acofarma (Madrid, Spain), and sodium phosphate monobasic and citric acid from Sigma-Aldrich (Darmstadt, Germany). Commercial lubricants K-Y® Jelly (Johnson & Johnson Lda., Porto Salvo, Portugal) and Durex® Play Original (Reckitt Benckiser Portugal, S.A., Lisboa, Portugal) were purchased from a local pharmacy. Universal Placebo, a hydroxyethylcellulose-based gel, was manufactured in-house as previously described [148].

Ethyl ether, methanol, acetone and dimethyl sulfoxide (DMSO) were purchased from Merck Millipore (Billerica, MA, USA). Dichoromethane (DCM) was acquired from ThermoFisher Scientific (Waltham, MA, USA). 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was purchased from Sigma-Aldrich and N-hydroxysuccinimide (NHS) from Merck Millipore (Burlington, MA, USA). Cyanine7.5 amine (Cy7.5) was acquired from Lumiprobe (Hunt Valley, MD, USA).

Caco-2 colorectal cells (clone C2BBe1) were purchased from ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle medium (DMEM) was acquired from Lonza (Basel, Switzerland), fetal bovine serum (FBS), penicillin-streptomycin and trypsin-EDTA from

Invitrogen (Carlsbad, CA, USA). Triton X-100 was purchased from SPI-Chem (West Chester, PA, USA) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) from Sigma-Aldrich.

All other materials, solvents and reagents were of analytical grade or equivalent.

3.2. Production and optimization of thermosensitive enemas

Studies for establishing a base thermosensitive enema were initially performed by dispersing enough amounts of poloxamer 407 in 20 ml ultrapure water under magnetic stirring and room temperature in order to yield different final concentrations (10, 15, 18, 20, 25 and 30% (*w/v*)). Dispersions were placed at 4-8°C overnight for allowing compete dissolution of the polymer and obtaining translucent solutions before volume being completed to 25 mL in volumetric flasks. Upon establishing the optimal concentration of poloxamer 407, different possible formulations were prepared using 10 mM phosphate buffer or 10 mM citrate, at pH 7, as solvent. The incorporation of 0.1% (*w/v*) sorbic acid (preservative) or 2-5% (*w/v*) glycerin (humectant) was also tested, these concentration values were selected taking into account the limit defined by WHO [44]. All excipients were dissolved in buffers prior to poloxamer 407 incorporation. Sorbic acid required mild heating and vigorous stirring in order to completely dissolve. In order to prepare NPs-in-thermo, a concentrated mixture of 20 mM phosphate buffer, 4% (*w/w*) glycerin and 30% (*w/v*) poloxamer 407 was prepared as described above before being mixed in equal parts with a suspension of NPs (see preparation details further in this section). Final NPs-in-thermo contained 0.5% (*w/v*) NPs, 15% (*w/v*) poloxamer 407 and 2% (*w/v*) glycerin in 10 mM phosphate buffer.

3.3. Characterization of thermosensitive enemas

3.3.1. Sol-gel transition temperature

The temperature at which transition from liquid state to gel state (sol-gel transition temperature) occurred was measured using two different procedures: magnetic bar

immobilization technique and rheological measurements. The magnet immobilization technique was performed as reported by Yun *et al.* [149]. The apparatus used for this technique is presented in **Figure 8.** A transparent beaker (25 mL) containing a magnetic bar (0.3 x 1.5 cm, 10 g) was used as a recipient for thermosensitive enemas. Samples were placed in a low-temperature water bath (approximately 10 °C). Temperature of the thermosensitive enemas was monitored using a thermometer immersed into the beaker. The enema was then heated at a constant rate (2 ºC/min) while the magnetic bar was kept under stirring at 40 rpm. Upon immobilization of the magnetic bar, the temperature displayed on the thermometer was registered and used as the sol-gel temperature.

The sol-gel transition was further characterized using rheological measurements. Details are provided in the next sub-section.

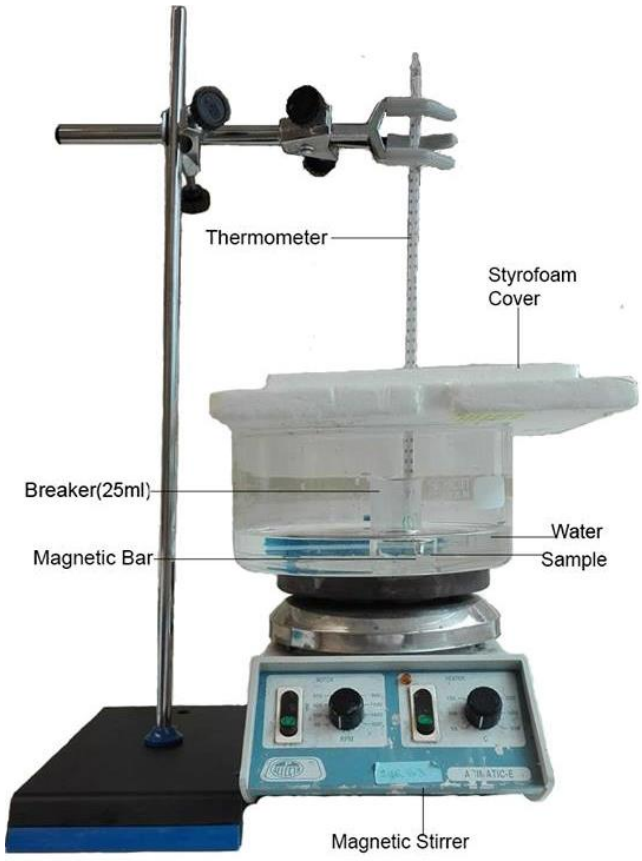

Figure 8: Layout of the setup used for performing the magnetic bar immobilization technique. A beaker of 25 mL containing the sample and a magnetic bar was placed in the water bath at a temperature of approximate of 10 ºC. The thermometer was placed in direct contact with the sample to be tested and the magnetic bar kept under stirring (40 rpm). Then, the temperature was increased at an approximate rate of 2 ºC/min until immobilization of the magnetic bar. The sol-gel transition temperature was considered as the temperature at which the bar stopped.

3.3.2. Rheological properties

The measurement of the rheological properties was carried out with a Kinexus Pro equipment (Malvern, UK) using a cone-plate geometry (diameter 40 mm; angle 4º). Initially, a dynamic stress sweep of samples was conducted at a constant angular frequency (1 Hz) over a shear strain of 0.01% to 10,000%, and an oscillatory frequency sweep test was performed between 0.01 Hz and 10 Hz at a constant shear rate of 0.1%, in order to assure that all further determinations were in the linear viscoelasticity region [150].

The sol-gel transition temperature of tested samples was determined by the dominant modulus shift from viscous to elastic with increasing temperature. Experiments were performed by submitting samples to a temperature ramp between 10 ºC and 40 ºC at 1 ºC/min (shear strain of 0.1 and frequency of 1 Hz). The sol-gel transition temperature was determined as exemplified in the **Figure 9**.

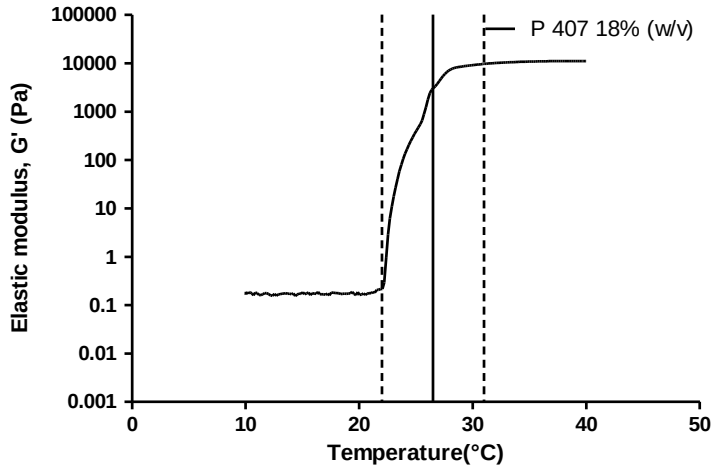

Figure 9: Example of the procedure undertaken to identify the sol-gel transition temperature point. The vertical full line equidistant of the dashed lines (corresponding to the beginning and ending of the elastic modulus shift) was used to determine the sol-gel transition temperature.

The viscoelastic properties of tested samples were further determined by measuring the shear viscosity profile between shear rates of 0.1 and 100 s^{-1}.

3.3.3. Measurement of pH

The pH of different samples was measured with a pHmeter ST 1000 (ebro Electronic, Ingostadt, Germany) at room temperature by direct immersion of the electrode of the equipment.

3.3.4. Osmolality

Osmolality values were determined by vapor pressure osmometry using a Vapro vapor pressure osmometer 5520 (Wescor Inc, Logan, UT, USA). The device was calibrated with standard solutions of 100, 290, and 1,000 mmol/kg. Samples were typically measured as whole. Whenever not possible, samples were diluted 1:1 with unionized water and the obtained osmolality values multiplied by the dilution factor.

3.4. Synthesis of PLGA-Cyanine7.5

The synthesis of fluorescently labeled PLGA with Cyanine 7.5 (Cy7.5) was conducted using click chemistry. The protocol used in this work was adapted from das Neves *et al*. [61]. Briefly, the carbodiimide group of EDC reacts with the terminal carboxyl group of PLGA forming an unstable intermediate compound. Then, NHS reacts with the previous compound to promote the reaction with the amine group of Cy7.5. In practice, PLGA-COOH (0.012 mmol, 528 mg) and Cy7.5 (0.018 mmol, 14.75 mg) were coupled in DCM with excess EDC (0.06 mmol, 11.50 mg) and NHS (0.06 mmol, 6.90 mg), under stirring for 2 h at room temperature. PLGA-Cy7.5 was then precipitated with 30 mL of ice-cold ethyl ether/methanol (1:1, *v/v*) and collected by centrifugation (5 min, 1,500 ×*g*). The polymer was re-dissolved in 1mL of DCM and the precipitation process repeated twice as described, with 6 mL of ice-cold ethyl ether/methanol (1:1, *v/v*) in order to remove residual EDC and NHS. Then the solution was dried under vacuum at room temperature until constant mass (approximately 48 h).

3.5. Production and characterization of nanoparticles

3.5.1. Production of nanoparticle

PLGA-based NPs were produced by nanoprecipitation by adapting a previously described protocol [9], and described in the **Figure 10**. The polymer was initially dissolve

in 1 mL of acetone and was injected gently into 10 mL of aqueous solution of 0.1% (*w/v*) poloxamer 407 under magnetic stirring (200 rpm) using a 100 µL pipette. The dispersion was left under stirring for 3h to enable the partial evaporation of the organic phase. Then, the dispersion was concentrated and washed twice with 10 mL of ultrapure water by centrifugation at 1,500 rpm for 15 min using Amicon Ultra-15 Centrifugal Filter Units (Merck Millipore) with a molecular weight cut-off (MWCO) of 100 kDa. Fluorescent NPs were similarly by partially replacing PLGA with PLGA-Cy7.5.

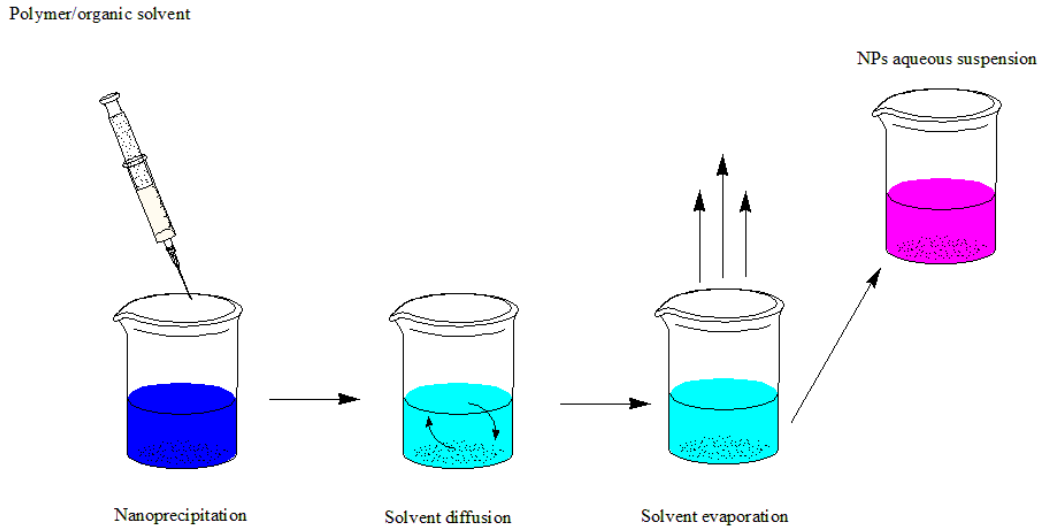

Figure 10: Schematic description of nanoprecipitation method used to produce PLGA nanoparticles. The polymer is dissolved in acetone and injected into aqueous phase. The rapid diffusion of the solvent within the water leads to the precipitation of the polymer in the form of NPs, followed by partial or total evaporation of the solvent

3.5.2. Characterization of nanoparticle

NPs were tested for size, polydispersity index (PdI) and zeta potential. The mean hydrodynamic diameter and the PdI were determined by Dynamic Light Scattering (DLS), while the zeta potential was tested by laser Doppler anemometry (LDA), using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). All of the assays were performed after 100-times dilution of the purified NP dispersion with 10 mM sodium chloride. The morphology of NPs was assessed by Transmission Electron Microscopy (TEM) using a JOEL JEM 1400 (JOEL, Tokyo, Japan). NPs were diluted 100-times using ultrapure water and observed upon treatment with a contrasting agent and placed into a grid of nickel.

3.6. *In vitro* cell studies

3.6.1. MTT reduction assay

The MTT reduction assay was used to evaluate the potential cytotoxicity of thermosensitive enemas (with or without NPs) or NPs alone to Caco-2 cells (passages 90-98). This assay measures metabolic activity of the cells following potential toxic challenge [151]. For that, cells were seeded on 96-well plates at a density of 10^4 cells/well in 200 µL of complete DMEM. After 24h of incubation, the medium was removed. Then, cells were treated with different formulation (thermosensitive enemas with or without NPs or NPs alone) in complete DMEM. Treatments with complete medium only and 2% (*v/v*) Triton X-100 in complete medium were used as positive and negative controls, respectively. Commercial lubricants (Durex® Play Original, K-Y® Jelly and Placebo Universal) were also tested for comparison purposes. All conditions were tested in triplicate. After incubation for 24h, cells were treated with 200 µL of MTT solution (0.5 mg/mL in complete DMEM). After 4h incubation, the MTT solution was removed and the formazan crystals, resulting from the reduction of MTT by viable cells, were solubilized using 200 µL of DMSO. The plates were placed in an orbital shaker at 100 rpm for 15 min in the dark at room temperature. Finally, the absorbance at 570 nm and 630 nm (used for subtraction of unspecific absorption) was measured using a Synergy Mx microplate reader (BioTek, Winooski, VT, USA). After subtracting background reading (*i.e.*, absorbance values at 570 nm of wells without any initial cells), cell viability was determined according to the following equation:

$$Cell\ Viability\ (\%) = \frac{Final\ absorbance\ value\ of\ sample}{Final\ absorbance\ value\ of\ positive\ control} \times\ 100$$

3.6.2. LDH release assay

In order to complement the previous technique, another assay was performed to assess the potential cytotoxicity of the NPs-in-thermo (with or without NPs) or NPs alone

in Caco-2 cells (passages 90-98). A lactate dehydrogenase (LDH) release assay was used to measure the integrity of the cell membrane. For that, cells were seeded on 96-well plates at a density of 10^4 cells/well in 200 µL of complete DMEM. After 24h of incubation, the medium was removed and cells were treated with different formulation (thermosensitive enemas with or without NPs, NPs alone) in complete DMEM. Treatments with complete medium only or 2% (*v/v*) Triton X-100 in complete medium were used as low and high controls, respectively. Commercial lubricants were again tested for comparison purposes. After incubation for 24h, 96-well plates were centrifuged at 250xg for 10 minutes. Then 100 µL of supernatants were transferred to another plate and 100 µL of a mixture for LDH detection commercial kit (Takara Bio Inc., Shiga, Japan) was added and incubated for 30 minutes. Finally, the absorbance at 490 nm and 690 nm (used for background deduction) was measured using a Synergy Mx microplate reader. After subtracting background reading (*i.e.*, absorbance values at 490 nm of wells without any initial cells), cell viability was determined according to the following equation recommended by the manufacturer of the assay kit::

$$Cytotoxicity\ (\%) = \frac{Experimental\ value - Low\ control}{High\ control - Low\ controle} \times\ 100$$

For the two assay the cell used were caco-2 colorectal cells (clone C2BBe1, passages 87-98) which were kept in DMEM supplemented with 10% (*v/v*) FBS, 100 U/mL penicillin and 100 µg/mL streptomycin (further referred to as complete DMEM). Cells were kept in a cell culture incubator (Binder, Tuttlingen, Germany) at 37 °C and 5% CO_2, under a water saturated atmosphere. Cells were sub-cultured every 4-5 days, using trypsin–EDTA as detaching agent.

3.7. *In vivo* distribution and retention of nanoparticles

A preliminary *in vivo* study was conducted using a mouse model in order to evaluate the distribution and retention of NPs following intrarectal administration as either NPs-in-thermo or dispersed in PBS (pH 7.0). Fluorescent NPs were used and *in vivo* tracking conducted using near infrared (NIR) imaging.

The methodology used in this work has been recently described in detail [9]. All experiments were approved by the Animal Welfare Body at i3S (reference BSm_2017_10) and conducted under FELASA and the European Directive 2010/63/EU guidance. Animal handling and rectal administration procedures were performed by Rute Nunes (PhD student, i3S). Briefly, 10-12 weeks old ICR mice were fasted for 24 h and submitted to rectal flushing with a water enema (200 mcL) using a micropipette equipped with plastic tip at 30 min before administration. Abdominal fur was trimmed using a hair clipper in order to reduce interference during imaging. Five hundred micrograms of fluorescent NPs dispersed in a volume of 100 μL of either thermosensitive enema or PBS were administered to a depth of approximately 0.5 cm using a positive displacement pipette equipped with plastic tip under brief anesthesia (5% inhalational isoflurane). Animals were restrained in a head-down position for one minute before recovering from anesthesia and left unrestrained in individual cages lined at the floor with absorbent paper. Animals were euthanized at 15 min and 2 h post-administration and evaluated by NIR imaging using an IVIS Lumina LT system (Perkin Elmer, Waltham, MA, USA) at wavelength values of excitation and emission of 745 nm and 813 nm, respectively. After whole body imaging, the entire colon of each animal – including upstream cecum and the last part of the jejunum and downstream rectum – was collected following necropsy, placed into a petri dish and analyzed by NIR imaging. Paper lining from cages was also assessed. Intensity of the NIR fluorescent signal was determined from collected images using the Living Image® software v. 4.4 (Caliper, Hopkinton, MA, USA) and expressed as total radiant efficiency. These values provide a semi-quantitative measure of the retention of NPs in colorectum [9]. Also, the extension of the fluorescent signal across the total length of the colorectum (estimated from grey-scale photographs superimposing the radiant signal maps) was calculated manually with the aid of the segmented line tool from ImageJ software (v. 1.51j8, NIH, Rockville, MD, USA). Results were expressed as coverage length percentage.

3.8. Statistical analysis

Numerical data are presented as mean ± standard deviation (SD) values of triplicate experiments, unless otherwise stated. Analysis was performed using Student's

t-test for two group comparisons or one-way ANOVA with post-hoc Tukey's HSD test for multiple group (three or more) comparisons. Data were processed using Prism 5.01 software (GraphPad Software, La Jolla, CA, USA) and $p<0.05$ was accepted as denoting significance.

4. Results and discussion[2]

4.1. Defining the optimal features of thermosensitive enema

Before initiating the actual experimental work of producing thermosensitive systems based on poloxamer 407, we first set out to define ideal features that should govern the optimization process and overall properties of enemas. These were established by taking into consideration previous work on rectal microbicide formulation (thoroughly reviewed in [6, 13]) and are summarized in **Table 2**. Enemas should possess characteristics that allow suitable rectal administration without damage, discomfort or excessive leakage. Many of these features are directly or indirectly related with properties such as the sol-gel transition temperature and time, pH, osmolality, viscosity or volume.

Table 2: Optimal properties of thermosensitive enemas.

Properties	**Optimal value/range**	**Reason/intended purpose**
Sol-gel transition temperature	30-35 ºC	Allows maintaining liquid state at typical storage temperature, while jellying at just below body temperature
Sol-gel transition time	1-5 min	Should be slow enough for favoring initial fast distribution but sufficiently fast for promoting retention upon jellification
pH	7.0-7.5	Complies with natural colorectal fluid pH values; particularly relevant for safety reasons
Osmolality	≈ 300 mOsm/Kg	Complies with natural colorectal fluid osmolality values; particularly relevant for safety reasons
Viscosity (in the gel state)	200-6,000 cps	In line with products available in the market, namely rectal lubricants
Volume (considering human use)	5-35 mL	Values complying with minimum and maximum volumes previously described as enabling adequate and tolerable administration

[2] Data presented and discussed in this section have been selected for oral presentation at the next HIV Research for Prevention Conference in Madrid, Spain (October 2018) as "Melo M., Nunes R., Sarmento B., das Neves J., Nanoparticles-in-thermosensitive enemas as potential vehicles for microbicide development". The presenting author (Mélanie Melo) has also been granted a full scholarship by the Organizing Committee to attend this conference.

Selection of a sol-gel temperature value just below 37 ºC is quite obvious taking into consideration the typical value at the rectum [152]. The selected range of 30-35 ºC is also compatible with the thermosensitive range observed for poloxamers and various others polymers used in pharmacy [153, 154]. In the case of poloxamer 407, a triblock copolymer with a center block of hydrophobic polypropylene oxide (PPO) linked to two hydrophilic polyethylene oxide (PEO) arms at each end, it presents reversible gelation properties close to physiological temperatures depending on concentration. When the temperature increases, the polymer molecules aggregate into micelles with spherical form and featuring PPO dehydrated cores. The sol-gel transition occurs by micellization displaying a centered cubic structure as describe by Liu *et al*. [138]. As for the sol-gel transition time, selected values were mostly empiric but in line with other works in the field of thermosensitive systems used for rectal drug delivery [120].

Another relevant feature concerns pH values. Apart from possibly governing the absorption of the drugs [36], it is important that physiological values are maintained, particularly due to the possibility of the onset of toxicity. Thus, pH should be within the range of 7.0-7.5 [155, 156]. Osmolality can affect the safety of rectal products and, in particular, contribute to enhanced transmission of HIV [41]. Indeed, the WHO alongside other international organizations advises that the osmolality of rectal lubricants should be equal or less than 1,200 mOsm/kg[41, 44]. Ideally, maximum osmolality should be even lower. Therefore, osmolality for an ideal enema should be as much as possible compliant with physiological values (280-300 mOsm/Kg) [157]. Another property of interest is the viscosity of the gel that is formed upon sol-gel transition, namely in determining user's acceptability and the ability to retain within the rectum. Selecting an ideal value or range of values is, therefore, not an easy task. Various popular gel products available in the market feature viscosity between 200-6,000 cps at different shear rates [42, 44]. A viscosity value or range for the fluid state was not defined but should tentatively be within what is empirically considered as a liquid, particularly regarding Newtonian behavior. This should be in line with the need for wide distribution soon after rectal administration and before jellification [158].

Although not addressed specifically in characterization studies described in the following, volume of enemas should also be bear in mind when designing such dosage forms. For example, results from a small trial suggest that volumes up to 35 mL would be acceptable to the majority of men when considering lubricants [159]. Another factor that is really important related to the necessity to administer a volume that can potentially cover all the colorectal area requiring protection. This volume has been estimated to be at least around 5 mL [160].

Given the general ideal features presented above and the polymer selected to produce a thermosensitive enema (which is also recognized for its safety record, namely for rectal use [161]), efforts were taken in order to establish an optimized basic formulation. Initial steps comprised the study of the sol-gel transition properties of poloxamer 407 at different concentrations in water, which is detailed over the next sub-section.

4.2. Development and optimization of a thermosensitive enema base

Formulation work began by testing the effects of different concentrations of poloxamer 407 in water on relevant features of the resulting tentative thermosensitive enema bases. Properties such as the sol-gel transition temperature and time, viscosity, osmolality, and pH were evaluated.

4.2.1. Sol-gel transition

The sol-gel transition is one of the critical parameters to evaluate in thermosensitive drug delivery systems, since it is primordial to determine the temperature, as well as the required amount of time, at which the gelation process occurs. Two techniques were used in this work: the magnetic bar immobilization technique described by Yun *et al.* [149], which allows a rougher evaluation of the gelation point yet closer to the reality that is required to be observed, namely the formation of a consistent mass that can promote retention. The second technique involved the determination of rheological parameters that allow obtaining precise information regarding a more physical definition, namely the

point at which the elastic modulus dominates over the viscous modulus [139]. This shift is useful for indicating the formal onset of the gelation process, even if not necessarily translating into the immediate formation of what can be macroscopically perceived as a gel (*i.e.*, a body that cannot flow by the simple action of its weight over a time-frame in the order of seconds) [162].

As previously stated, the selected thermosensitive polymer was poloxamer 407 and various concentrations in purified water were tested: 15%, 18%, 20%, 25% and 30% (*w/v*). As expected, a negative correlation between the polymer concentration and the sol-gel transition temperature was observed, as determined by the magnetic bar immobilization technique (**Figure 11**). Higher concentrations led to a reduction of the sol-gel transition temperature from 18 °C for 30% (*w/v*) to 29 °C for 18% (*w/v*). At 15% (*w/v*), it was not possible to immobilize the magnetic bar even past 37 °C thus indicating that the gel configuration could not be achieved. Obtained results are in general agreement with the scientific literature (**Figure 11**), although with some deviations, namely at the concentration of 15% (*w/v*). These can be caused by differences in technical procedures (*e.g.*, heating rates, magnetic bar size and rotation speed, volume of samples, etc.), which are common since the assay is not standardized. Another possibility leading to variability may be differences between samples and batches of poloxamer 407, even if provided by the same manufacturer/supplier [163].

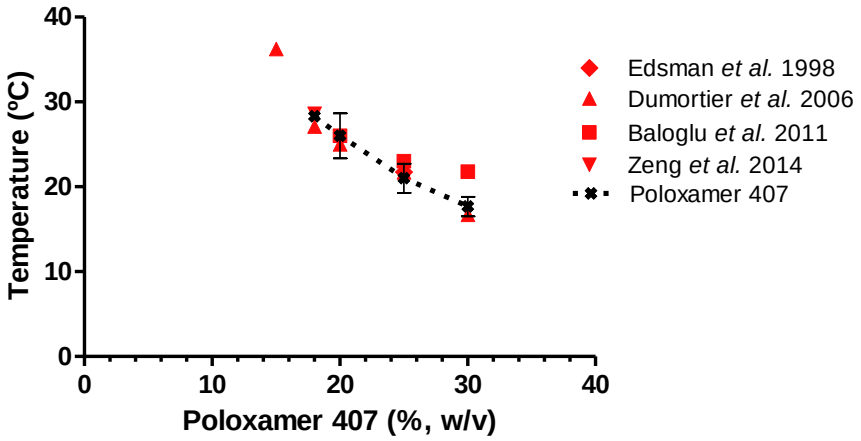

Figure 11: Sol-gel transition temperatures for different concentrations of poloxamer 407 in water as determined by the magnetic bar immobilization technique. Results are presented as mean ± SD (n=3). Data (mean results) obtained from other works are presented in red for comparison purposes: Edsman *et al.* 1998 [164]; Dumortier *et al*. 2006 [165]; Baloglu *et al*. 2011 [166]; and Zeng *et al*. 2014 [167].

Next, sol-gel transition properties were determined by rheological measurements. Before testing the shift of moduli as induced by temperature increase, a preliminary assay was performed in order to verify that measurements were performed in the linear viscoelasticity zone. This step is essential in order to guarantee reliability of obtained results [168]. For this, both frequency and strain sweeps were performed using poloxamer 407 solutions over the range of 10-30% (*w/v*), as shown in **Figure 12**. Common values of 1 Hz for frequency and 0.1% for strain were chosen in order to comply with the linear viscoelasticity zone for all concentrations of poloxamer 407.

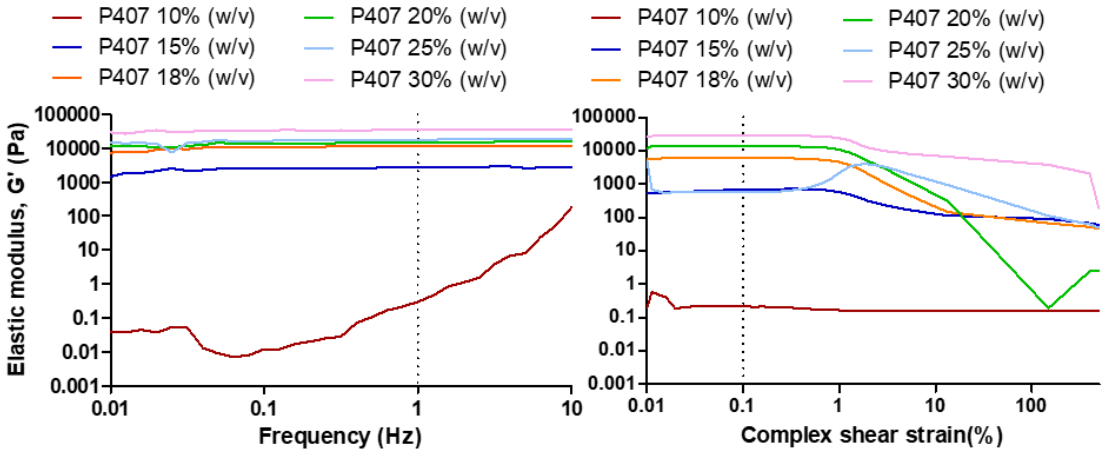

Figure 12: Typical strain and frequency sweep tests for different concentrations of poloxamer 407 (indicated as P407). Data were used for the determination of linear viscoelasticity zone. Values of frequency and strain selected for future analysis were 1 Hz and 0.1%, respectively (highlight by vertical dash lines in graphs).

Rheological tests were then performed by measuring the variation of elasticity and viscosity moduli with temperature. Results are presented in **Figure 13**. The same negative correlation observed for the magnetic bar immobilization technique was verified, with sol-gel transition temperatures varying from 13 °C to 30 °C when poloxamer 407 concentrations were decreased from 30% to 15% (*w/v*). Interestingly and contrasting with the previous technique, it was possible to observe a sol-gel transition for poloxamer 407 at 15% (*w/v*) using rheological measurements. As discussed above, this may indicate the formation of a weak, low consistency gel that was not macroscopically apparent and therefore may not be adequate for enhancing colorectal retention. Differences obtained for the two techniques may be justified by the obvious diverse nature of the assays. However, one particular issue may be highlighted, namely the reduced sample volume used in rheological measurements. This can facilitate heating transfer to and within the

sample, thus leading to a more homogeneous and almost immediate sol-gel transition. Importantly, obtained values are in line with those described in the literature using rheological measurements (**Figure 13**). There are still some differences, which could be related with the type of geometry used and/or by protocol used for the identification of the sol-gel transition. Indeed, shift between moduli typically occurs over a range of temperature, which leads to different interpretations of rheograms. Moreover, the exact methodology is not described in the overwhelming majority of the scientific literature thus making comparison troublesome.

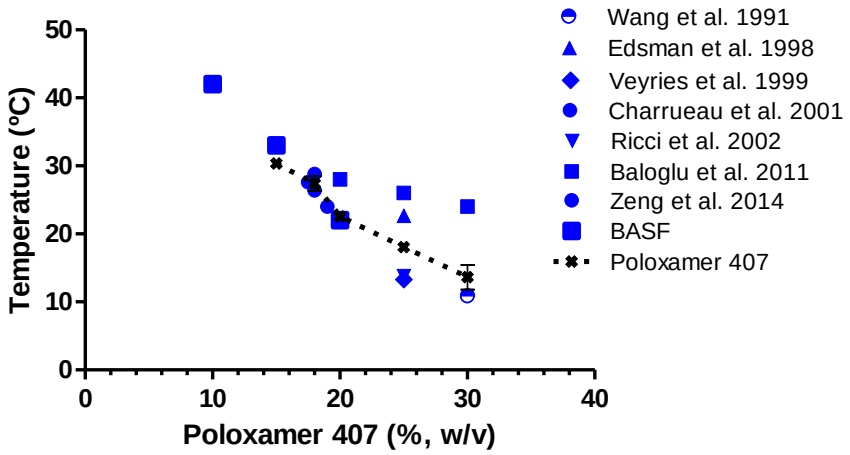

Figure 13: Sol-gel transition temperatures for different concentrations of poloxamer 407 in water as determined by rheological measurements. Results are presented as mean ± SD (*n*=3). Data (mean results) obtained from other works are presented in blue for comparison purposes: Wang *et al.* 1991 [169]; Edsman *et al.* 1998 [164]; Veyries *et al.* 1999 [170]; Charrueau *et al.* 2001 [171]; Ricci *et al.* 2002 [172]; Baloglu *et al.* 2011 [166]; Zeng *et al.* 2014 [167]; and BASF SE Chemical Corporation [173].

In addition to the temperature, the time required for poloxamer 407 samples to undergo sol-gel transition once at 37 °C was also determined. This was performed by using the same apparatus described for magnetic bar immobilization technique. The temperature of the water bath was fixed at 37 °C before introducing the beaker containing samples and the magnetic bar. Sol-gel transition time values were similar for all concentrations, namely 2.9 ± 0.8 min, 2.8 ± 0.5 min, 2.7 ± 0.3 min and 2.6 ± 0.1 min for 18%, 20%, 25% and 30% (*w/v*) poloxamer 407, respectively. Interpretation of such values should be made carefully since they reflect the apparatus setup and may not accurately reflect the behavior of such samples in a more realistic scenario, namely in the colorectum, in which factors such as spreading, heat transfer rates and stress are quite different. However, data seems

to indicate that an initial time lag is necessary for the formation of a gel. This may be beneficial from an initial distribution point of view.

Taking into account the results presented above for sol-gel transition properties of poloxamer 407 in water, the concentration that best fitted the values considered as optimal for thermosensitive enemas (**Table 2**) was 18% (*w/v*). Although mean sol-gel transition temperature values (29 ± 1 °C and 27.4 ± 1.1 °C for magnetic bar immobilization technique and rheological measurements, respectively) were slightly below the one defined for the lower optimal threshold (30 °C), these may still be considerable as suitable for the development of thermosensitive enemas. Approaches such as the use of mixtures of different poloxamers or poloxamers with other polymers could be useful in order to modulate sol-gel transition temperature, as previously described [174-176]. However, due to the limited amount of time available to execute the initial work plan, a decision not to test such possibilities was made and we proceeded with further work with the 18% (*w/v*) poloxamer 407 concentration.

Next step towards the establishment of a thermosensitive enema was the study of the influence of including excipients of interest, which are typically use in rectal dosage forms, in the sol-gel transition properties of aqueous 18% (*w/v*) poloxamer 407. Excipients included a buffer system (to maintain pH at 7), glycerin (a humectant) and sorbic acid (a preservative). Tested buffer systems were based on citric acid and sodium phosphate and used at 10 mM. Glycerin was chosen due to its wide use as a humectant; however, two different but low concentrations (2% and 5% (*w/v*)) were tested in order to comply with WHO recommendations for osmolality of rectal lubricants and related products. As for sorbic acid, this preservative was tested at 0.1% and included in the study due to its previously reported favorable safety profile when tested in vaginal microbicide products [177, 178].

When initiating these additional studies, however, a mishap occurred as there was the need to change between Kolliphor® P407 batches from WPWJ584B to GNA18121C. The former was produced at the BASF production site in Ludwigshafen, Germany, while the previous was from the USA plant of BASF at Geismar, LA. Although supplied as the

same product (Kolliphor® P407) featuring the same technical specifications, the two batches presented several differences, namely in MW (Annex 1). More important, the two different samples presented different sol-gel transition properties as determined in the present work. Similar observations have been reported recently by Fakhari *et al.* [163] for different batches of poloxamer 407, and could be related with variations in the heterogeneity of the copolymer as originated by diverse production processes, leading to a disparity of impurities that interfere with micellization [179]. In practical terms, the solution of poloxamer 407 from the new batch at the concentration previously selected as optimal (18% (*w/v*)) jellified at room temperature. A quick test, however, showed that poloxamer 407 from new batch was able to yield results at 15% (*w/v*) for sol-gel transition properties that were similar to those observed for the old batch at 18% (*w/v*) (**Table 3**). Thus, the 15% (*w/v*) concentration of poloxamer 407 from the new batch was selected for proceeding with the remaining work. Although advisable, preliminary work regarding the optimization of poloxamer 407 concentration was not repeated due to time constrictions.

Going back to the objectives and results of the studies regarding the inclusion of different excipients, no relevant changes were observed in terms of sol-gel transition of the plain 15% (*w/v*) poloxamer 407 solution (**Table 3**). Apart from binary combinations (poloxamer 407 + one excipient), some ternary mixtures were also considered. Poloxamer 407 at 15% (*w/v*) + glycerin at 5% (*w/v*) in 10 mM phosphate buffer (pH 7.0) also presented similar sol-gel transition properties. However, sorbic acid precipitated when included in phosphate buffer (pH 7.0) and this mixture was therefore considered unsuitable for further development. Further research into the literature revealed that sorbic acid indeed presents low solubility in neutral to alkaline solutions, as well as decreased antimicrobial activity [180]. A possible alternative to consider as preservative could be parabens. However, its use in microbicides is not consensual, namely regarding safety, and therefore the work was continued with a non-preserved system. In the future and in order to ensure the microbiological stability of a potential commercial product, sterilization and single use applicators could be considered for guarantying quality.

Table 3: Changes in sol-gel transition temperature and time of poloxamer 407 at 15% (*w/v*) in water with the incorporation of different excipients. Results are presented as mean ± SD (n=3). No statistical differences were found ($p \geq 0.05$).

Excipients	Sol-gel transition temperature (ºC)		Sol-gel transition time (min)
	Magnetic bar immobilization technique	Rheological measurements	
-	29 ± 2	29.9 ± 1.1	3.1 ± 0.3
Citric acid (10 mM, pH 7.0)	29 ± 1	28.2 ± 0.3	3.1 ±0.7
Sodium phosphate (10 mM, pH 7.0)	30 ± 1	28.1 ± 0.5	2.9 ± 0.7
Glycerin 2% (*w/v*)	30 ± 3	28.4 ± 0.6	3.3 ± 0.3
Glycerin 5% (*w/v*)	29 ± 2	28.0 ± 0.6	2.6 ± 0.2
Sorbic acid 0.1% (*w/v*)	29 ± 1	28.7 ± 0.9	3.2 ± 0.8
Sodium phosphate (10 mM) + glycerin 5% (*w/v*)	29 ± 1	28.9 ± 0.8	3.2 ± 0.3
Sodium phosphate (10 mM) + sorbic acid 0.1% (*w/v*)	-	-	-

4.2.2. Viscosity characterization

Apart from sol-gel transition properties, another important feature of a potential thermosensitive enema is viscosity. Flow behavior of polymeric liquid and semi-solid systems is known not only to affect behavior *in vivo* (*e.g.*, administration, leakage from the rectum) but also production processes (*e.g.*, transfer between vessels, packaging) or physicochemical stability (*e.g.*, sedimentation of materials in suspension upon storage) [181]. The viscosity of different concentrations of poloxamer 407 (still performed with the old batch) were evaluated at 20 °C. As expected, higher concentrations of poloxamer 407 led to an increase in viscosity as shown in **Figure 14**. Results are only presented at 40 s^{-1} for clarity and comparability since this value of shear rate is consider as physiological relevant for the colorectal compartment [158]. Samples with a concentration up to 20% (*w/v*) showed Newtonian behavior, which is typical of liquids, while at higher concentration this shifted towards non-Newtonian, shear thinning behavior, consistent with a gel. Indeed, samples of 25% and 30% had profiles similar to that of commercial gels (K-Y® Jelly and Durex® Play Original) or the well characterized Universal Placebo [182], thus reinforcing the gel-like consistency even at room temperature. From an empirical point of view, samples with a concentration up to 20%

(*w/v*) presented viscosities that could be compared to those of water (0.001 Pa s) or olive oil (0.067 Pa s), which are seen as typical liquids [183].

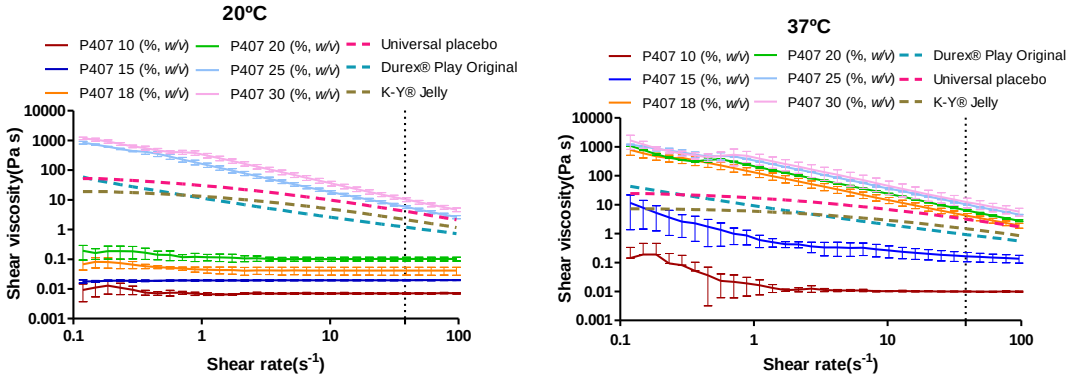

Figure 14: Shear viscosity profiles at 20 ºC and 37 ºC for poloxamer 407 (10%, 15%, 18%, 20%, 25% and 30% (*w/v*)). Commercial lubricants (K-Y® Jelly and Durex® Play Original) and Universal Placebo were included for comparison purposes. Results are presented as mean ± SD (*n*=3). Vertical dash line highlights ciscosity values at the shear rate of ≈40 s^{-1}.

When viscosity profiles were analyzed at 37 °C (**Figure 15**), it was possible to verify a dramatic change for the concentrations of 15%, 18% and 20% (*w/v*) in poloxamer 407 with viscosities of 0.16 ± 0.06 Pa s, 4.17 ± 0.80 Pa s and 6.33 ± 0.40 Pa s at a shear rate of 40 s^{-1}, respectively. Significantly, concentrations of 18% and 20% shifted towards a non-Newtonian, shear thinning behavior, while presenting profiles that were closer to higher poloxamer 407 concentrations, commercial lubricants and the Universal Placebo. This indicates that, at such concentrations, gels of suitable consistency may be formed upon administration and thus potentially contribute to enhanced retention. At the lowest concentration in water (10%), poloxamer 407 presented a small variation in viscosity profile but still closer to a liquid than a gel, which seems to reflect the inability to undergo sol-gel transition as previously shown. The effect for poloxamer 15% (*w/v*) was more undefined, again suggesting that this concentration is likely on the boundary of the thermosensitive ability of the copolymer.

Overall, viscosity data confirms the suitability of the concentration of 18% (*w/v*) for poloxamer 407 from the old batch in water for developing the base of the thermosensitive enema. However, due to the change between batches described before, the work proceeded with the concentration of 15% (*w/v*) of poloxamer 407 from the new batch. Again, viscosity profiles were interchangeable for both batches at considered concentrations.

The influence of adding excipients to the plain 15% (*w/v*) poloxamer 407 solution in water on the viscosity profile at 37 °C was further studied. Results for viscosity at a shear rate of 40 s^{-1} and 37 ºC are presented in **Table 4**. Profiles were generally unaffected, although a small decrease in viscosity was apparent for glycerin at 2% (*w/v*) or sorbic acid. Still, results are within the same order of magnitude and are unlikely to reflect any practical differences among considered formulations. The ternary mixture of 15% (*w/v*) poloxamer 407, 10 mM phosphate buffer (pH 7.0) and 2% (*w/v*) glycerin also did not show notable changes despite a statistical difference being observed. Moreover, viscosity values at a shear rate of 40 s^{-1} for poloxamer 407-based samples were in line with those for commercial lubricants (Durex® Play Original: 0.92 ± 0.01 Pa s; K-Y® Jelly: 1.46 ± 0.02) or the Universal Placebo (3.11 ± 0.00).

Table 4: Viscosity determined at 37 ºC and shear rate of 40 s^{-1} of mixtures of 15% (*w/v*) poloxamer 407 with different excipients. Results are presented as mean ± SD (*n*=3). (*) indicates a statistically significant difference from the 15% (*w/v*) poloxamer 407 formulation ($p<0.05$).

Excipients	**Shear viscosity (Pa s)**
-	2.33 ± 0.45
Citric acid (10 mM, pH 7.0)	2.67 ± 0.02
Sodium phosphate (10 mM, pH 7.0)	2.15 ± 0.33
Sorbic acid 0.1% (*w/v*)	1.17 ± 0.12
Glycerin 2% (*w/v*)	0.58 ± 0.12*
Glycerin 5% (*w/v*)	2.22 ± 0.81
Sodium phosphate (10 mM, pH 7.0) + glycerin 2% (*w/v*)	2.85 ± 0.55

4.2.3. Osmolality

Osmolality plays a paramount in safety of lubricants and should be within values considered compatible with rectal use [40-43]. Preliminary studies of osmolality allowed to evaluate the variation of this parameter with the increase of the concentration poloxamer 407 (old batch) dissolved in water. Osmolality values at concentrations of 10%, 15%, 18%, 20%, 25% and 30% (*w/v*) were 34.7 ± 1.5, 67.0 ± 1.7, 90.3 ± 3.0, 136.7 ± 6.1, 181.3 ± 10.3 and 362.0 ± 40.0 mOsm/kg, respectively, thus indicating that a positive correlation between concentration of poloxamer 407 and osmolality. Different results were observed for old and new batches of poloxamer 407 at concentrations of 18%

(*w/v*) and 15% (*w/v*), respectively (**Table 5**). However, overall osmolality values were still low and comparable. As for the addition of excipients, only glycerin had a significant impact on osmolality. These values are in line with the recommendation of the WHO for glycerin levels in lubricants [44]. Even at 5% (*w/v*) glycerin, values are much lower than the maximum threshold defined by this international body and clearly below the value determined for K-Y® Jelly (3,215 ± 17 mOsm/kg). In order to preserve near isosmolality values for enemas developed in this work, the concentration of 2% (*w/v*) glycerin was chosen for further studies. Indeed, the results of 15% (*w/v*) poloxamer 407 in phosphate buffer (10 mM, pH 7.0) added with 2% (*w/v*) glycerin presented values of osmolality that were comparable to those of the Universal Placebo (308 ± 6 mOsm/kg).

Table 5: Osmolality values for mixtures of 15% (*w/v*) poloxamer 407 with different excipients. Results are presented as mean ± SD (*n*=3). (*) indicates a statistically significant difference from the 15% (*w/v*) poloxamer 407 formulation ($p<0.05$).

Excipients	**Osmolality (mOsm/kg)**
-	67 ± 2
Citric acid (10 mM, pH 7.0)	99 ± 11
Sodium phosphate (10 mM, pH 7.0)	92 ± 4
Sorbic acid 0.1% (*w/v*)	72 ± 7
Glycerin 2% (*w/v*)	297 ± 39*
Glycerin 5% (*w/v*)	686 ± 18*
Sodium phosphate (10 mM, pH 7.0) + glycerin 2% (*w/v*)	335 ± 1*

4.2.1. pH

Following the previous characterization studies and in order to validate the final pH of the tentative enemas, the pH was formally measured. Preliminary experiments with the old poloxamer 407 batch showed that this polymer yield nearly neutral solutions (6.9-7.1) within the concentration range of 10-30% (*w/v*). The further addition of the excipients had no effect in pH except for sorbic acid (**Table 6**). This shift in pH is consistent with the pKa of sorbic acid, approximately 4.8.

Taking into account all of the previous studies and the optimal profile of a thermosensitive enema (**Table 2**), the basic formulation further used in this work was composed of 15%

(*w/v*) poloxamer 407 and 2% (*w/v*) glycerin in 10 mM phosphate buffer at a final pH of 7.0. This resulted in a thermosensitive enema with the following properties: sol-gel transition temperature of ≈29 ºC, sol-gel transition time ≈3 min, viscosity of ≈3 Pa s at a shear rate of $39s^{-1}$, osmolality of ≈335 mmol/Kg and a pH of ≈7.0.

Table 6: Values of pH for mixtures of 15% (*w/v*) poloxamer 407 with different excipients. Results are presented as mean ± SD (*n*=3). (*) indicates a statistically significant difference from the 15% (*w/v*) poloxamer 407 formulation ($p<0.05$).

Excipients	**pH**
-	7.1 ± 0.1
Citric acid (10 mM, pH 7.0)	7.0 ± 0.1
Sodium phosphate (10 mM, pH 7.0)	7.0 ± 0.1
Sorbic acid 0.1% (*w/v*)	4.9 ± 0.4*
Glycerin 2% (*w/v*)	6.9 ± 0.1
Glycerin 5% (*w/v*)	6.8 ± 0.1
Sodium phosphate (10 mM, pH 7.0) + glycerin 2% (*w/v*)	7.4 ± 0.4

4.3. Characterization of nanoparticles

PLGA-based NPs were considered for incorporation in thermosensitive enemas. This type of NPs are widely used for drug delivery purposes in research and are typically considered as potentially safe for mucosal use [20]. Preparation of NPs was made according to a nanoprecipitation technique previously described [9]. Two polymers of PLGA with different MW (inherent viscosity midpoint of 0.2 dl/g for PLGA 5002A and 0.4 dl/g for PLGA 5004A) were tested. Both types of PLGA yield NPs with similar colloidal properties (**Table 7**). The negative values of zeta potential of the nanoparticles are attributed to PLGA carboxyl end group presence. As no particular feature of either polymer was considered more or less relevant to the work, PLGA 5004A was selected, essentially due to the lower PdI values of NPs produced with it. More important and taking into account the later objectives of the project, which include tracking of NPs in mice by NIR imaging, PLGA was modified with a NIR fluorescent probe. Cy7.5 was

conjugated to the polymer by carbodiimide chemistry by a protocol that was previously established [61]. Although no formal physicochemical characterization of the modified polymer was made, NIR imaging demonstrated that the final material produced substantial quanta. PLGA-Cy7.5 was used along plain PLGA at different ratios in order to producing fluorescent NPs (**Table 7**). The criterion for selecting a ratio of PLGA-Cy7.5 was the size of obtained NPs. The selected ratio was thus 0.02% (*w/v*) of PLGA-Cy7.5 since it did not lead to important changes of diameter of NPs.

Table 7: Values for hydrodynamic diameter, polydispersity index (PdI) and zeta potential of different PLGA-based NPs. Results are presented as mean ± SD (n=3) (*) indicates a statistically significant difference from PLGA 5004A NPs (p<0.05).

Composition of NPs	**Diameter (nm)**	**PdI**	**Zeta potential (mV)**
PLGA 5002A	169 ± 7	0.150 ± 0.020*	-16.0 ± 0.5*
PLGA 5004A	171 ± 1	0.105 ± 0.010	-12.3 ± 1.3
PLGA 5004 A + 0.02% (*w/w*) PLGA-Cy7.5	179 ± 2	0.122 ± 0.007	-11.5 ± 0.8
PLGA 5004 A + 0.07% (*w/w*) PLGA-Cy7.5	194 ± 2*	0.136 ± 0.023	-12.1 ± 1.4
PLGA 5004 A + 0.09% (*w/w*) PLGA-Cy7.5	234 ± 1*	0.175 ± 0.008*	-12.3 ± 1.0

Size confirmation and morphological analysis of plain and fluorescent NPs was performed by transmission electron microscopy (TEM). Representative images are shown in **Figure 15**. It was possible to observe that the diameters of round particles were in range with those determined by DLS, as well as the apparent low size dispersion of samples. Differences in the electronic density of images from plain and fluorescent NPs were apparent and this could be related with the presence of Cy7.5. However, further investigation into this matter is required.

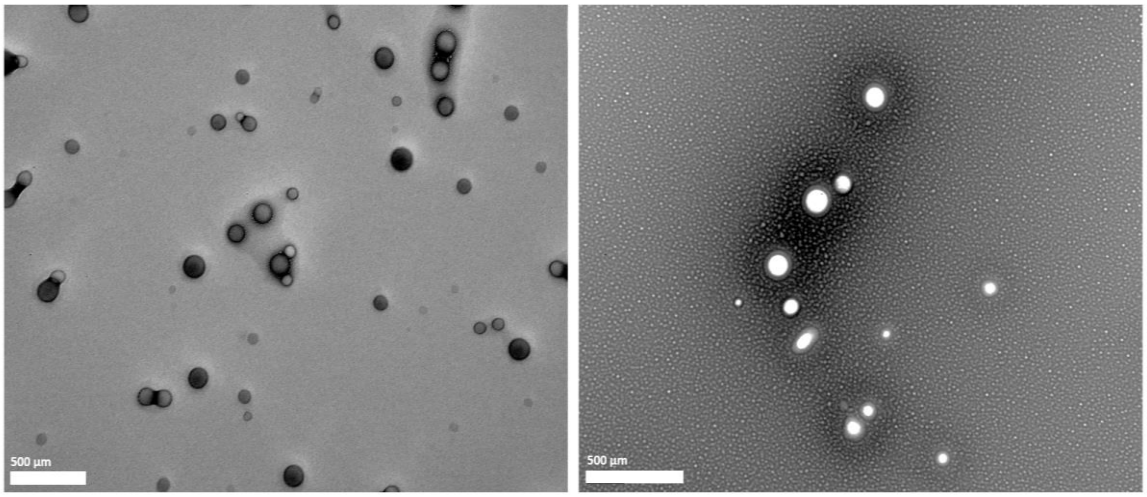

Figure 15: TEM images of plain PLGA 5004A NPs (left) and fluorescent PLGA5004A + 0.02% (*w/w*) PLGA-Cy7.5 NPs (right).

4.4. Characterization of NPs-in-thermo

Various NPs-in-thermo were produced by simply incorporating plain of fluorescent NPs in the basic thermosensitive enema previously optimized. In order to establish the influence of NP incorporation into the thermosensitive enema, particles at different concentrations (0.01% (*w/v*), 0.1% (*w/v*) and 1% (*w/v*)) were added and the characterization of obtained systems performed as previously described (**Table 8**). The effects of incorporating NPs at all considered concentrations was minimal for most properties. However, a decrease in sol-gel transition time was noted for 0.1% (*w/v*) and 1% (*w/v*) of NPs but values remained within the pre-defined optimal range. The osmolality was also changed with increasing amount of NPs. Values, however, were still quite below the maximum threshold of 1,200 mOsm/kg established by the WHO for rectal lubricants [41, 44]. Overall, changes induced by the incorporation of NPs in thermosensitive enemas are limited and, thus, obtained NPs-in-thermo were considered for more studies.

Table 8: Properties of thermosensitive enema upon the incorporation of different concentrations of plain NPs. Results are presented as mean ± SD (n=3). (*) indicates a statistically significant difference from the formulation without NPs (p<0.05).

	Enema base			
	Without NPs	**0.01% (*w/v*) NPs**	**0.1% (*w/v*) NPs**	**1% (*w/v*) NPs**
Sol-gel transition temperature by magnetic bar immobilization technique (ºC)	30 ± 1	31 ± 2	28 ± 1	27 ± 1
Sol-gel transition temperature by rheological measurements (ºC)	28.9 ± 0.8	29.6 ± 1.2	28.3 ± 0.5	27.9 ± 0.2
Sol-gel transition time (min)	2.8 ± 0.6	2.2 ± 0.2	1.6 ± 0.4*	1.6 ± 0.1*
Osmolality (mOsm/kg)	335 ± 2	348 ± 5	430 ± 9*	470 ± 4*
pH	7.4 ± 0.4	7.1 ± 0.0	6.9 ± 0.2	7.2 ± 0.0
Viscosity @ 20 ºC (Pa s) and 40 s^{-1}	0.019 ± 0.001	0.021 ± 0.001	0.024 ± 0.001*	0.031 ± 0.002*
Viscosity @ 37 ºC (Pa s) and 40 s^{-1}	2.9 ± 0.5	2.5 ± 0.6	2.0 ± 0.5	2.6 ± 0.4

4.5. *In vitro* studies

Upon establishment and physicochemical characterization of a basic thermosensitive enema and NPs-in-thermo formulations that met criteria pre-defined as optimal, the work continued by evaluating potential cellular toxicity. Two assays, namely those based on MTT reduction and LDH release, were used to evaluate cell metabolic activity and membrane integrity, respectively. Initial tests with NPs (plain or containing PLGA-Cy7.5) showed little potential to cause damage to Caco-2 cells at considered concentrations after 24 h incubation (**Figure 16**). Viability was around or above 100% up to 0.2% (*w/v*) NPs; similarly, cytotoxicity was roughly 20% or lower. Values well above 100% in viability for some concentrations of both PLGA and PLGA-Cy7.5 NPs were also noted. These may be related with poor washing of cells during assays, which contribute to non-specific absorption during final readings because of the alteration of the viscosity of medium. Combined, these data appear to confirm the previously established good cell

compatibility of PLGA-based NPs [184]. Significantly, the incorporation of PLGA-Cy7.5 did not change the behavior as compared to NPs composed by PLGA alone.

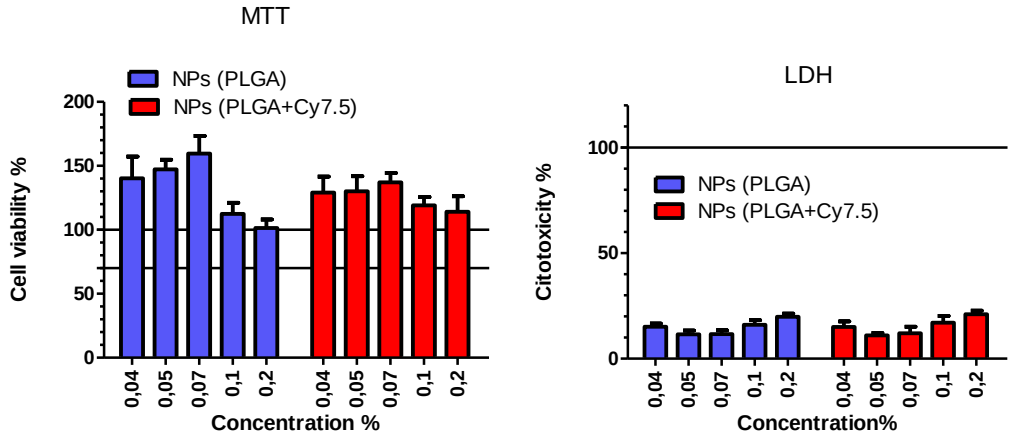

Figure 16: Cell viability (left; as assessed by the MTT reduction assay) and cytotoxicity (right; as assessed by the LDH release assay) of NPs, with and without PLGA-Cy7.5, up to 0.2% (*w/v*) in Caco-2 cells after 24h incubation. Results are presented as mean ± SD (*n*=2).

Next, viability of Caco-2 cells exposed to different dilutions of either basic thermosensitive enema or NPs-in-thermo was studied. Results are presented in **Figure 17**. In general, viability was maintained above the minimum threshold of 70% in the range of 1.75-20% dilution of enemas, which is considered as potentially indicating good biocompatibility as per ISO guidelines [185]. However, the basic thermosensitive enema deviated from such results for the 10-20% dilutions. These unexpected results do not seem to have a straightforward explanations but problems with sample preparation could be implicated. Thus, new experiments should be performed in order to clarify whether or not cell viability is being affected. The addition of both plain or PLGA-Cy7.5 modified NPs did not seem to affect viability up to 20% dilution when considering a maximum of 1% (*w/v*) of particles in NPs-in-thermo. Also significantly, all enema formulations presented results that were similar or improved as compared to commercial lubricants or the Universal Placebo. Cytotoxicity of the various enemas was also evaluated (**Figure 18**). No important concerns regarding both basic thermosensitive enema and NPs-in-thermo were noted, with mean cytotoxicity values being maintained below 15%. A slight increase in the case of NPs containing PLGA-Cy7.5 was, however, apparent but still within values considered as normal for this type of samples and assay [148]. Results for cytotoxicity as assessed by the LDH release assay were also in line or better than those for commercial

lubricants or the Universal Placebo, thus confirming the good biocompatibility profile of proposed enemas. An increase in cytotoxicity for the highest concentration tested for K-Y® Jelly was noted, which could be directly related to the unacceptable osmolality of such product (3215.1 ± 17.4 mOsm/kg). Overall, NPs-in-thermo containing up to at least 1% (*w/v*) of either plain or fluorescent NPs seem to be biocompatible and thus adequate for further evaluation.

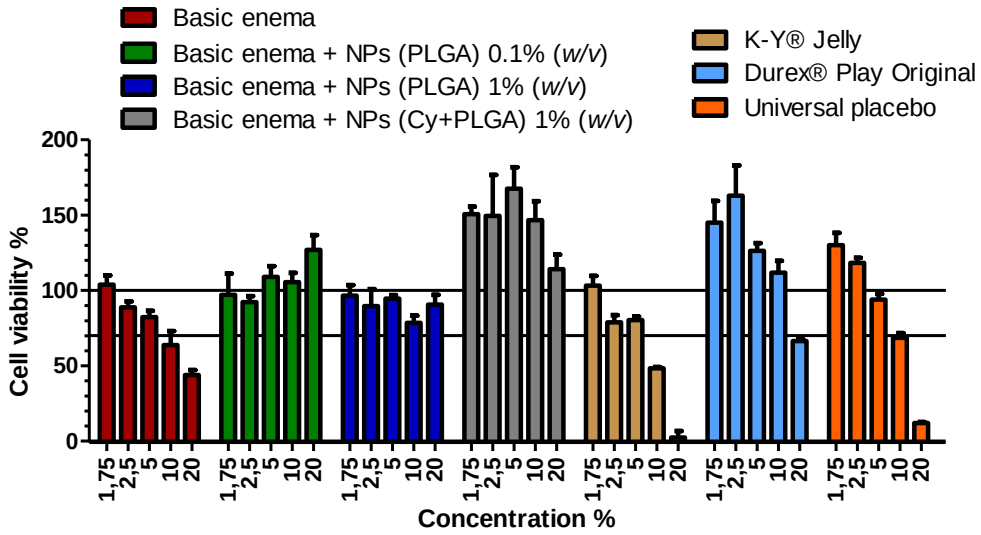

Figure 17: Cell viability, as assessed by the MTT reduction assay, of basic thermosensitive enema and different NPs-in-thermo up to 20% dilution in Caco-2 cells after 24h incubation. Data for commercial lubricants (K-Y® Jelly and Durex® Play Original) and the Universal Placebo are also presented for comparison purposes. Results are presented as mean ± SD (*n*=2).

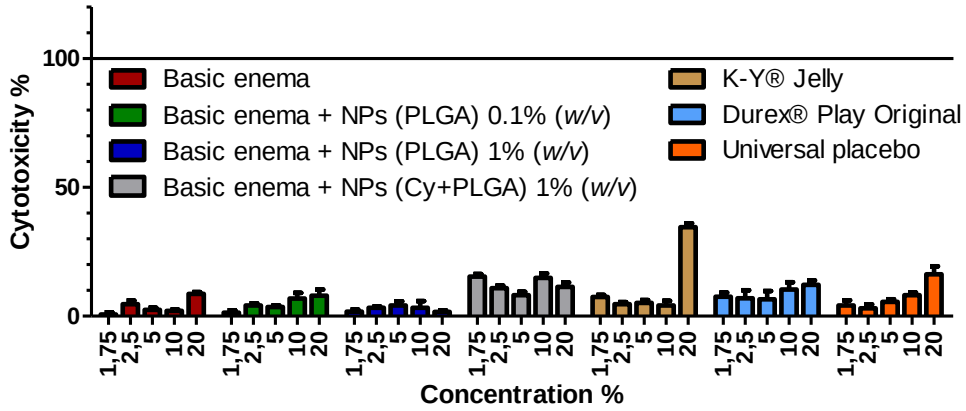

Figure 18: Cytotoxicity, as assessed by the LDH release assay, of basic thermosensitive enema and different NPs-in-thermo up to 20% dilution in Caco-2 cells after 24h incubation. Data for commercial lubricants (K-Y® Jelly and Durex® Play Original) and the Universal Placebo are also presented for comparison purposes. Results are presented as mean ± SD (*n*=2).

4.1. Preliminary evaluation of the distribution and retention of nanoparticles in mice

An exploratory *in vivo* study was conducted in order to establish experimental conditions for future work in order to assess the potential value of developed NPs-in-thermo. Although lacking definitive status, experiments also provided interesting hints and trends that will be considered as guides. The fluorescent signal from NPs in aqueous suspension as well as incorporated into the thermosensitive enema was confirmed using the IVIS Lumina LT system under the same conditions used in subsequent *in vivo* and *ex vivo* imaging (**Figure 19**). Although previous experiments have been performed using enemas containing either particles at 0.01% 0.1% or 1% (*w/v*), animal studies were conducted using particles at 0.5% (*w/v*). This adjustment was based on previous data from our group and related with the optimization of the NIR signal obtained using the IVIS Lumina LT system [9]. Imaging of NPs in PBS or incorporated into the basic thermosensitive enema indicated that an adequate NIR signal could be obtained. The signal was intense enough but not too bright to saturate NIR filters and resulting images.

Figure 19: NIR imaging of (A) fluorescent NPs at 2% (*w/v*) in PBS (pH 7.4), (B) fluorescent NPs at 0.5% (*w/v*) in PBS, (C) fluorescent NPs at 0.5% (*w/v*) in thermosensitive enema, (D) plain NPs at 0.5% (*w/v*) in PBS, and (F) plain NPs at 0.5% (*w/v*) in thermosensitive enema. Representative images for duplicate wells are presented.

Whole body imaging (**Figure 20**) was unable to yield any distinguishable abdominal fluorescent signal as previously described in a work from our group [9]. Differences in the nature of NPs (Cy7.5 was associated in a non-covalent fashion), as well as in the total amount of administered particles (900 μg vs. 500 μg in the present work), may explain this outcome. Still, erratic fluorescent signal was observed around the anal zone and mostly at immediately after administration (not shown) or following 15 min, which indicates that moderate leakage occurred particularly soon after administration in both experimental groups.

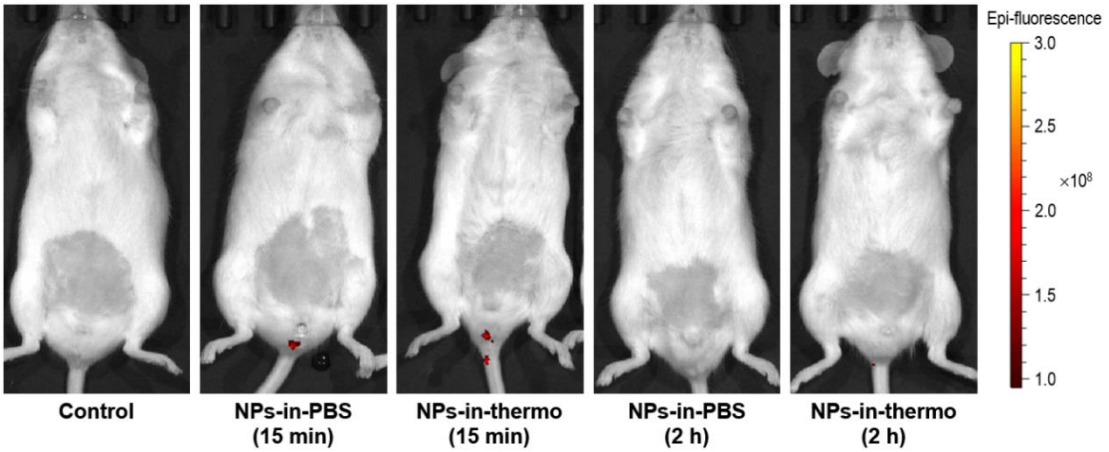

Figure 20**:** Representative whole body imaging of mice positioned in supine position after 15 min and 2 h of rectal administration of either fluorescent NPs dispersed in PBS (NPs-in-PBS) or in thermosensitive enema (NPs-in-thermo). Image of an animal treated with thermosensitive enema containing non-fluorescent NPs, referred to as 'Control', is included for reference. Heat map scale ranges from 0.95×10^8 to 3.0×10^8 p cm^2 s^{-1} μW^{-1}.

The lower section of GIT, ranging from the last few centimeters of the ileum to the anus, was isolated and analyzed by NIR imaging. Results are presented in **Figure 21** Fluorescence signal was clearly identifiable from 15 min to 2 h in animals treated with fluorescent NPs, either included in the thermosensitive enema or dispersed in PBS (**Figure 21A**). NPs-in-thermo presented a distribution pattern that was more restricted to the last third of the colon/rectum than NPs in PBS at 15 min post-administration. However, NPs-in-thermo appear to have consistently provided good distribution and retention in the distal colorectum, contrasting with more uneven coverage at the terminal colon and rectum. This effect was more pronounced after 2 h of administration.

Semi-quantitative evaluation of retention, as estimated from the total radiant efficiency signal from tissues, showed no significant differences between NPs-in-thermo and NPs dispersed in PBS at both timepoints (**Figure 21B**). Even if a trend for higher retention of NPs could be apparent for NPs-in-thermo at 2 h, these results need to be further confirmed, namely by using a larger number of animals. The relative extension of the colon covered by NPs (**Figure 21C**) was tentatively lower (although without significance) in the case of NPs-in-thermo at 15 min post-administration. This suggests that the enema may undergo relatively fast sol-gel transition upon instillation into the rectum. Also, relatively higher viscosity of the thermosensitive enema as compared to PBS even at room temperature may already limit retrograde colon distribution. However, this pattern appears to have shifted at 2 h, which could indicate that the thermosensitive

system may prolong the presence of NPs in the colorectum. Overall and keeping in mind all the limitations of this preliminary study, our data seems to suggest that the use of the proposed thermosensitive enema as a vehicle for the rectal delivery of NPs may mildly reduce initial distribution as compared to the administration in PBS, although approximately up to at least one third of the total colon length could still be covered. As for retention, the thermosensitive enema seems to provide an efficient way to reduce leakage of NPs. Additional work regarding these issues is in progress.

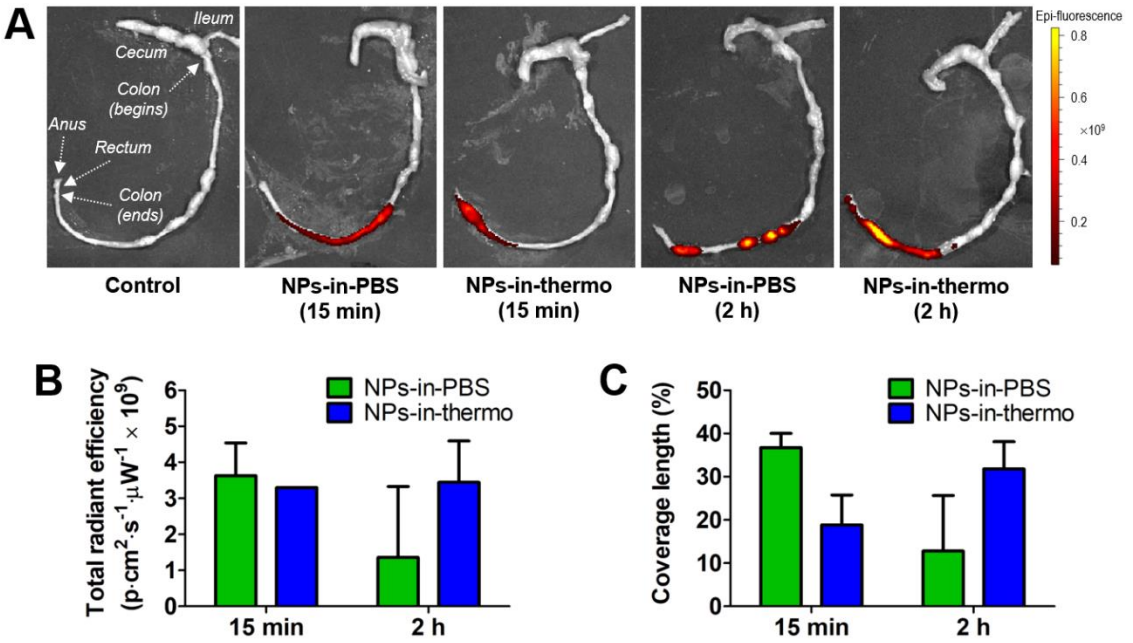

Figure 21: Qualitative and semi-quantitative assessment of the distribution and retention of fluorescent NPs using NIR imaging. (A) Typical fluorescence signal from excised lower gastrointestinal tract at 15 min and 2 h following administration of either fluorescent NPs dispersed in PBS (NPs-in-PBS) or in thermosensitive enema (NPs-in-thermo). Signal is from PLGA-Cy7.5 at 745 nm excitation and 813 nm emission. Heat map scale ranges from 0.06×10^9 to 0.83×10^9 p cm2 s-1 μW-1. Image of the control contains identification of relevant anatomical sites for reference purposes. (B) Semi-quantitative evaluation of the amount of NPs associated with excised tissues and (C) estimation of the relative length of the colon presenting fluorescence signal as a measure of the total coverage percentage of NPs. Columns represent mean values and bars the standard error of the mean (n=2), except for NPs-in-thermo at 15 min (n=1). No significant differences were observed between different groups at the same time point (p<0.05; Student's t-test) were noted between NPs-in-thermo and NPs-in-PBS when comparing results for total radiant efficiency or coverage length at each time point.

5. Conclusions and future work

The main objective of this work comprised the development of NPs-in-thermo that could be further considered for the development of rectal anti-HIV microbicide products. In order to do so, a poloxamer 407-based formulation was established and thoroughly characterized for relevant properties. In particular, the basic thermosensitive enema – 15% (*w/v*) poloxamer 407 and 2% (*w/v*) glycerin in 10 mM phosphate buffer at a final pH of 7.0 – was shown able to undergo sol-gel transition at values of temperature and time that were within pre-established ranges considered adequate for rectal administration. Moreover, viscosity studies at room and body temperature indicated that the optimized basic thermosensitive enema could be useful in enhancing colorectal distribution and retention. Other properties relevant to the overall safety of the basic thermosensitive enema, namely osmolality and pH, were within a range considered adequate for rectal use. Indeed, *in vitro* cell studies confirmed the good biocompatibility of the formulation.

PLGA-based NPs were also prepared using either the plain polymer or modified with a fluorescent probe (Cy7.5) by using a click chemistry protocol. The incorporation of NPs into the basic thermosensitive enema (NPs-in-thermo) did not change notably the previous physicochemical and *in vitro* biological properties of the parental formulation and were therefore considered for further animal studies. Importantly, NPs-in-thermo containing 0.5% (*w/v*) fluorescent NPs were shown to possess adequate NIR signal to be used for *in vivo* and *ex vivo* NIT imaging. Preliminary experiments demonstrated that NPs-in-thermo were able to produce appreciable fluorescence signal that allowed its *in vivo* and *ex vivo* tracking. Data suggested that the incorporation of NPs into the thermosensitive enema could result in suitable distribution and enhanced retention following rectal administration to mice. Still, more definitive results are needed in the future to fully assess the utility of NPs-in-thermo, particularly in the context of anti-HIV microbicide development.

Despite all reported findings, future work could help strengthening presented data. Apart from the above mentioned complementary *in vivo* experiments, which are already

in progress, other issues should be addressed. For example, additional studies concerning the microbiological stability of the basic thermosensitive enema formulation, namely by including one or more suitable preservatives, could be helpful. Also, the incorporation of other excipients (*e.g.*, stabilizers, antioxidants, coloring agents) that could be interesting for more specific formulations is another interesting topic to address. In all cases, the ground physicochemical and biological features should be maintained. Another aspect deserving more insight concerns the characterization of PLGA-Cy7.5. Although the protocol used for its production has been previously establish, the degree and stability of bonding of Cy7.5 to PLGA, as well as the characteristics of the fluorescent polymer (*e.g.*, MW, solubility, fluorescent signal stability) should be assessed. These data could be helpful in the further optimization of the coupling protocol for obtaining PLGA-Cy7.5. Another issue relates to the stability of NPs-in-thermo upon storage. This type of studies were clearly outside the scope of the present work but one should recognize its paramount importance from a pharmaceutical development point of view. Future work is therefore required, particularly when considering more specific formulations already containing antiretroviral drugs. Finally, the safety of NPs-in-thermo requires expanded studies, both *in vitro* and *in vivo*. This aspect has been a hallmark of microbicide development, without which no product can move forward towards clinical testing.

Overall, NPs-in-thermo proposed in this work present the potential to be further developed and used as microbicide drug delivery platforms for rectal use.

6. References

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7. Annex 1

BTC
Chemical Distribution

Certificate of Analysis

BTC Europe GmbH

Fax No 021733347211

BTC Europe GmbH
CoA Receiver
Rheinpromenade 1

40789 Monheim am Rhein

Germany

2018-05-30
EN
BASF CORPORATION

Certificate No 5604
Page 1 of 3

Certificate of Analysis according to DIN 55350-18-4.2.2

Kollidon® P 407 Geismar

0,5KG Plastic bottle
Purchase Order/Customer Product#
5005931124
000000000050424592

Material	50424592
Order	3014646478 000010
Delivery	3190366436 000010
Lot	GNA18121C
Lot/Qty	1.000 KG
Total	1.000 KG
Transport	00000000000782804376

Characteristic/Method	UOM	Result	Specification
Water STI 10502	Weight %	0.48	<=0.75
Average Molecular Mass STI11065	g/mol	13971	10000-14600
Average Molecular Weight STI11065	g/mol	13971	9840-14600
pH Value (1:40) STI10119		6.7	5.0-7.5
pH Value (1:10) STI10119		6.7	5.0-7.5
Cloud Point, 10% in Water STI 3041	°C	> 100	>=100
Color (50:50 in MeOH) STI 3057	APHA	90	<=120
Residual EO STI11339	ppm	< 1	<=1
Residual PO STI11339	ppm	< 1	<=5
Residual 1, 4 - Dioxane STI11339	ppm	< 1	<=5
Clarity and color of solution STI11192		Pass, Clear and colorless	
Appearance of Solution (= Solution S) STI11616		Pass, Less than BY7	
Heavy Metals STI11199		Pass, Less than 0.002%	

The aforementioned data shall constitute the agreed contractual quality of the product at the time of passing of risk. The data are controlled at regular intervals as part of our quality assurance program. Neither these data nor the properties of product specimens shall imply any legally binding guarantee of certain properties or of fitness for a specific purpose. No liability of ours can be derived therefrom.

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BTC
Chemical Distribution

Certificate of Analysis

BTC Europe GmbH

Fax No 021733347211

BTC Europe GmbH
CoA Receiver
Rheinpromenade 1

40789 Monheim am Rhein

Germany

2018-05-30
EN
BASF CORPORATION

Certificate No 5604
Page 2 of 3

Certificate of Analysis according to DIN 55350-18-4.2.2

Kolliphor® P 407 Geismar

0,5KG Plastic bottle
Purchase Order/Customer Product#
5005931124
000000000050424592

Material	50424592
Order	3014646478 000010
Delivery	3190366436 000010
Lot	GNA18121C
Lot/Qty	1.000 KG
Total	1.000 KG
Transport	00000000000782804376

Characteristic/Method	UOM	Result	Specification
Butylhydroxytoluene STI11352	ppm	183	50-125
Sum of Etheylene and Diethylene Glycol STI11356		Pass, Less than 0.25 wt%	
Oxyethylene Wyandotte	Weight %	72.5	71.5-74.9
Unsaturation STI 3056	mEq/g	0.043	0.031-0.065
Residual Solvent STI11467		Meets USP Requirements	
Total Ash STI10523	%	0.3	<=0.4
Identification (IR) STI 10201		Matches Standard Scan	
Acid STI11715		Pass	
Residue on Ignition STI10523	Weight %	0.28	<=0.30
Congealing Temperature STI11712	°C	52	50-62
Arsenic Wyandotte	ppm	< 2	<=2
Retest Date		07/02/2019	

Manufacturing Date: 07/02/2016

*Ethylene Glycol,, <=620 ppm,, STI11713

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Chemical Distribution

Certificate of Analysis

BTC Europe GmbH

Fax No 021733347211

BTC Europe GmbH
CoA Receiver
Rheinpromenade 1

40789 Monheim am Rhein

Germany

2018-05-30
EN
BASF CORPORATION

Certificate No 5604
Page 3 of 3

Certificate of Analysis according to DIN 55350-18-4.2.2

Kolliphor® P 407 Geismar

0,5KG Plastic bottle
Purchase Order/Customer Product#
5005931124
000000000050424592

Material	50424592
Order	3014646478 000010
Delivery	3190366436 000010
Lot	GNA18121C
Lot/Qty	1.000 KG
Total	1.000 KG
Transport	00000000000782804376

Poloxamer 407 with 100 ppm BHT

The product meets current USP-NF, EP and JPE requirements.

The Residual Solvent Ethylene Glycol is not used in the manufacture, and historical analysis of the finished product has demonstrated that there is no potential for ethylene glycol to be present in the final product. Ethylene Glycol is tested only on random lots.

Manufactured at:
BASF Corporation
8404 River Rd
Geismar, LA 70734

THIS CERTIFICATE OF ANALYSIS HAS BEEN PRODUCED ELECTRONICALLY AND IS VALID WITHOUT A SIGNATURE.

This is a computer-generated document. No signature is required.

Certificate of Analysis according to DIN 55350-18-4.2.2

Kolliphor® P 407	Material	50254759
	Order	3011335106 000030
90KG Fibre drum	Delivery	3095045831 000030
Purchase Order/Customer Product#	Lot	WPWJ584B
Entregas Agosto 2015	Lot/Qty	90.000 KG
LUTR10	Total	90.000 KG
	Transport	SC-KR 73

Characteristic/Method	UOM	Result	Specification
Identification (IR) WQTM-358		Matches Standard Scan	
Appearance of Solution WQTM-616		Pass, Less than BY7	
Clarity and color of solution WQTM-192		Pass, Clear and colorless	
Acid WQTM-715		Pass, Red Solution	
pH value (25g/L in water) WQTM-020		6.8	5.0-7.5
pH value (100g/L in water) pH	pH-value	6.8	5.0-7.5
Average Molecular Weight WQTM-065	g/mol	12919	9840-14600
Oxyethylene Wyandotte	Weight %	72.5	71.5-74.9
Total Ash WQTM-195	Weight %	0.3	<=0.4
Residue On Ignition WQTM-195	Weight %	0.24	<=0.30
Unsaturation WQTM-056	mEq/g	0.039	0.031-0.065
Water WQTM-062	Weight %	0.05	<=0.75
Heavy Metals WQTM-199		Pass, Less than 0.002%	
BHT Stabilizer	ppm	101	50-125

The aforementioned data shall constitute the agreed contractual quality of the product at the time of passing of risk. The data are controlled at regular intervals as part of our quality assurance program. Neither these data nor the properties of product specimens shall imply any legally binding guarantee of certain properties or of fitness for a specific purpose. No liability of ours can be derived therefrom.

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Certificate of Analysis

Characteristic/Method	UOM	Result	Specification
Congealing Point WQTM-352	°C	54	50-62
APHA Color (50:50 in MeOH) WQTM-712		40	<=120
Cloud Point, 10% solution WQTM-057	°C	> 100	>=100
Residual Solvent WQTM-132		Meets USP Requirements	
Sum Ethylene and Diethylene Glycol USP-PROC		Pass, Less than 0.25 wt%	
1,4 Dioxane WQTM-713	ppm	0.1	<=5.0
Ethylene Oxide WQTM-339	ppm	0.1	<=1.0
Propylene Oxide WQTM-339	ppm	0.7	<=5.0
Arsenic WQTM-339		Pass, Less than 2 ppm	
NEXT INSPECTION DATE Wyandotte		20.01.2017	

Manufacturing Date: 20.01.2014

Poloxamer 407 with 100 ppm BHT

The product meets current USP-NF, EP and JPE requirements.

The Residual Solvent Ethylene Glycol is not used in the manufacture,

and historical analysis of the finished product has demonstrated that there is no potential for ethylene glycol to be present in the final product. Ethylene Glycol is tested only on random lots.

Manufactured at:
BASF Corporation
2 Pleasant View Ave
Washington, NJ 07882

THIS CERTIFICATE OF ANALYSIS HAS BEEN PRODUCED ELECTRONICALLY AND IS VALID WITHOUT A SIGNATURE.

Re-packaged by BTC Europe GmbH, Burgbernheim