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**Novel Polyurethane-based nanoparticles of infliximab to treat inflammation in an in-vitro intestinal epithelial barrier model**

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Abstract

In this study we examined the potential of novel biodegradable polymers of polyesterurethane (PU), and its PEGylated (PU-PEG) form as nanocarriers of Infliximab (INF), to treat inflammation in an in-vitro epithelial model. Nanoparticles (NPs) formulated were of average size of 200-287nm. INF loading of NPs (INF-NPs) resulted in an increase in size and zeta potential. No cytotoxicity was observed for any of the NPs. Cellular interaction and uptake of PU NPs were similar compared with polycaprolactone (PCL) NPs and significantly higher to Poly(lactic-*co*-glycolic) acid (PLGA) NPs. Cellular interaction was higher for corresponding PEG-NPs. INF-PU and INF-PU-PEG NPs showed a rapid rate and extent of recovery of the epithelial barrier function in inflamed Caco-2 cell monolayers and decreased cytokine levels in inflamed monocytes. Results obtained in this study are promising and the potential of PU and PU-PEG NPs for drug delivery and targeting to treat gastrointestinal inflammation warrants further investigation.

**Keywords:** Nanoparticles, Infliximab, IBD, Caco-2, Inflammation, TNF-$\alpha$, cellular uptake

## 1.0 INTRODUCTION

Inflammatory Bowel Disease (IBD) is a debilitating disease and has become one of the most common chronic inflammatory conditions with millions affected globally (Hepatology and 2015, n.d.; Qin, 2012). Although the cause of IBD remains poorly understood, a complex interaction between external environmental stress, genetic factors, intestinal microbial flora and mucosal immune responses have been identified

to be involved in the pathogenesis of IBD (de Mattos et al., 2015; Zhang and Li, 2014). These risk factors appear to initiate alterations in the intestinal epithelial barrier, allowing access of the luminal microbiome to the underlying intestinal mucosa, triggering the mucosal immune system and secretion of cytokines including TNF−$\alpha$ and IL-8 (Yadav et al 2016a). This causes aberrant and excessive cytokine responses, resulting in further damage to the intestinal epithelial barrier and mucosa, and development of acute intestinal inflammation. Treatment is usually initiated with conventional oral solid dosage forms of small molecule anti-inflammatory agents, corticosteroids and immunosuppressants, aminosalicylates, thiopurines, methotrexate which provide mainly symptomatic relief in mild to moderate cases. For patients who fail to respond to these conventional therapies, ultimately disease progression leads to chronic intestinal inflammation and to surgery in ~75% of patients, to remove the diseased tissue (Cader and Kaser, 2013; de Mattos et al., 2015; Littman and Pamer, 2011; Neurath, 2014). Numerous authors have attempted to enhance the effectiveness of the commercially available therapeutics such as Budesonide, Prednisolone, Dexamethasone, Rolipram, Tacrolimus, 5-ASA by developing micro/nanoparticle formulations aimed at targeting these therapeutics to the lower part of the GIT to the site of inflammation (Collnot et al., 2012; Hua et al., 2015; Lautenschläger et al., 2014; Xiao and Merlin, 2012).

Biodegradable NPs have been reported to show better adherence to the inflamed intestine and prolonged efficacy at a lower dose compared to the corresponding drug solutions, resulting in lower side effects and enhanced patient compliance. Lamprecht et al (Lamprecht et al., 2001) reported that rolipram-PLGA NPs administered orally to rats with induced colitis, reduced clinical activity score and sustained therapeutic activity for over 5 days, due to accumulation of the NPs in the tissues. The authors

further report that the efficacy of rolipram NPs was independent of size (332-474nm) and surface charge (-3 to-20mV). In an another study, oral or rectal administration of PLGA NPs of Tacrolimus, of size 107nm was found to be effective in mitigating colitis by selective and enhanced adhesion and penetration in the inflamed site (Lamprecht et al., 2005a, 2005b). Similarly, Pertuit et al (Pertuit et al., 2007) reported that 5-aminosalycylic acid conjugated PCL NPs with size of 200-330nm and near neutral charge was effective at a lower drug dose of 0.5mg/kg and showed an equivalent therapeutic efficacy compared to a 60-fold higher dose of 30mg/kg of corresponding drug solution in an in-vivo mouse colitis model.

Leonard et al reported that PLGA NPs of budesonide, of size 190-220nm, specifically adhered to the inflamed cells in an in-vitro 3D inflamed cell culture model, resulting in effectively reversing the inflammation, as observed by the recovery in the TEER and drop in the cytokines levels, compared to corresponding liposome formulations.

IBD therapy with conventional small molecule therapeutics including aminosalicylates, thiopurines, corticosteroids are characterized by cycles of relapsing and remitting mucosal inflammation, and ultimately disease progression leads to surgery. In comparison, biological therapeutics such as the anti-TNF-α mAbs, infliximab, adalimumab, golimumab, and a gut-selective antibody to $\alpha_4\beta_7$ integrin Vedolizumab, are reported to be highly effective in maintaining remission and promoting mucosal healing, thus reducing the rate of surgery, however these show life threatening side effects due to high systemic dose

To date no studies has been carried out exploring nanoformulations of anti-TNF-α mAbs to treat the inflamed tissues in IBD, designed for oral administration. The

sensitivity of mAbs to various physical, chemical and mechanical stresses present challenges to their formulation and retention of bioactivity (Alsaddique et al., 2016; Pabari et al., 2013). In addition, mAbs have been reported to be more stable in the colon i.e. at its site of action, compared to in the acidic environment of the stomach (Yadav et al 2016b).

The aim of this study was to examine the potential of novel biodegradable polymers of polyesterurethane (PU) and PU-PEG to act as nanocarriers of Infliximab (INF) to treat inflammation in an in-vitro epithelial barrier model. The cellular interaction and uptake of PU and PU-PEG NPs were first compared with those of poly(lactic-*co*-glycolic) acid (PLGA), PLGA-PEG, polycaprolactone (PCL) and PCL-PEG NPs. These polymers differ in hydrophobicities and can be arranged as PU > PCL > PLGA, hence were chosen for comparative evaluation (Mattu et al 2013). INF loading of NPs was carried out by electrostatic/hydrophobic interaction, to limit INF exposure to organic solvent, shear forces and high temperature of conventional microencapsulation processes. Cellular interaction and anti-inflammatory efficacy of the INF-NPs were examined in an in vitro inflamed epithelial barrier model and in inflamed monocytes.

## 2.0 MATERIALS AND METHODS

### 2.1 Materials

Poly-dl-lactide-co-glycolide 50:50 (PLGA); (Resomer RG 504;, 46-50 kDa) was purchased from Boehringer-Ingelheim (Ingelheim, Germany), PEGylated PLGA diblock containing 10% wt mPEG 5000 (PLGA-PEG) (5050 DLG;, 52.5 kDa) was purchased from Evonik Corporation (Birmingham, Alabama, US). Polycaprolactone (PCL, 48-90 kDa) was purchased from Sigma-Aldrich (Wicklow, Ireland). PEGylated

polycaprolactone (PCL-PEG), Polyurethane (PU), and PEGylated Polyurethane (PU-PEG) were synthesised in Prof Gianluca Ciardelli's lab (Alessandria, Italy).

PCL-diol (Mn = 2000 g/mol) was purchased from Acros Organics, Milan Italy. Hexamethylene diisocyanate (HDI), N-Boc-serinol (NS) and catalyst Dibutyltin dilaurate (DBTDL) were purchased from Sigma Aldrich (Milan, Italy). HDI was distilled before use. Poly (ethylene glycol) (average Mw 1,500 Da) was purchased from Fluka Analytical (Milan, Italy). Tween® 80, coumarin-6, solvents and reagents used were purchased from Sigma-Aldrich (Wicklow, Ireland and Milan, Italy). Remicade® (Infliximab®) was purchased from United Drug (Dublin, Ireland).

## 2.2 Methods

### 2.2.1 Polymer synthesis

The polyurethanes (NS-HC2000 and NS-HC2000E2000) used in this study were synthesised following a two-step synthesis procedure in inert atmosphere as previously described (Gentile et al., 2015; Mattu et al., 2013). The acronyms relate to their constituents: NS refers to the chain extender (n-Boc-serinol), H refers to the isocyanate (HDI), C2000 refers to PCL-diol, (Mn = 2000 g/mol), and E2000 refers to the macrodiol PEG (Mn 2000). Briefly, the macrodiol (PCL-diol for NS-HC2000 and a 70:30 ratio between PCL-diol and PEG for NS-HC2000E2000) was dissolved in anhydrous 1,2-dichloroethane (DCE) in an inert atmosphere. Distilled HDI was added at 2:1 M ratio with respect to the macrodiol, in the presence of a catalytic amount of DBTDL and stirred for 2.5h to allow the formation of an isocyanate-terminated pre-polymer. At the end of the pre-polymerisation reaction, N-Boc-serinol, was added at 1:1 M ratio with respect to PCL-diol, at room temperature and was left to stir overnight. After 16 h, the chain extension reaction was stopped by adding methanol. The polymer

solution was precipitated in petroleum ether, dried, and purified by dissolving in dimethylformamide (DMF) and precipitating in methanol in order to remove the low molecular weight impurities and residual catalyst.

PCL-PEG was also synthesized similarly, except that PEG was used as the chain extender at 1:1 M ratio with respect to PCL-diol.

**2.2.2 Polymer Characterisation**

The molecular weight (MW) of the polymer was assessed by Size Exclusion Chromatography (SEC, Agilent Technologies 1200 Series, USA), using a Refractive Index detector and two Waters Styragel columns (HT2 and HT4) conditioned at 35$^{o}$C. Tetrahydrofuran was used as mobile phase at a flow rate of 0.5 ml/min and an injection volume of 20 μl.

Attenuated Total Reflectance Fourier Transform Infrared (ATR-FT-IR) analysis of synthesised polymers was carried out in the spectral range from 4000 to 450 $cm^{-1}$ at 16 scans with a resolution of 4 $cm^{-1}$, using a Perkin Elmer Spectrum 100 equipped with an ATR accessory (UATR KRS5) with diamond crystal.

**2.2.3 Preparation of nanoparticles**

Nanoparticles (NPs) of PLGA, PCL, PU and their corresponding pegylated forms, were formulated using the solvent dispersion technique (Ramtoola et al., 2011). Polymer at 10, 20 or 55mg/ml, and coumarin-6 (C6) (at 0.3% w/w of polymer) was dissolved in acetone. This organic phase was added dropwise to aqueous Tween® 80 solution at 2% w/v (0.02% w/v for PU-PEG and PCL-PEG) using an organic/aqueous phase ratio of 0.1, while stirring at 480rpm. This mixture was stirred overnight to allow complete solvent evaporation. NPs were harvested by centrifugation

(Hettich Rotina 35R, DJB Labcare, Bucks, UK), washed three times, suspended in deionised water and stored at 4ºC. INF-NPs were prepared by incubating NP suspension (1mg/ml) with INF solution (1mg/ml in sterile water for injection) at 1:1 weight ratio of NP: INF for 2hours at 4ºC, under mild stirring. NP suspension, with or without INF were pre-frozen at -80°C and lyophilised at −56°C and 0.056 mbar (Labconco Freezone 6, model no. 7752030; Labconco, Kansas City, MO, USA).

### 2.2.4 Characterisation of nanoparticles

The size, polydispersity index (PDI) and zeta potential (ZP) of NPs dispersed in deionised water was measured by zetasizer (Nanoseries, Nano-ZS, Malvern Instruments, Worcestershire, UK) and reported as the average value (n=3) ± standard deviation. Lyophilised NPs were mounted on aluminium stub, were sputter coated with gold and examined using a scanning electron microscope (Tescan Mira, Warrendale, PA, USA) at a magnification of 35-50 kx. INF loading of NPs was assayed by UV spectroscopy and bioactivity of INF was measured using ELISA as previously published (Alsaddique et al., 2016).

### 2.2.5 Cell culture

Human colon epithelial cancer cell line, caco-2 cells (P55 to P65) were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% foetal bovine serum (FBS), 1% penicillin-streptomycin, 1% non-essential amino acids (NEAA) and incubated at 37°C in an atmosphere of 5% $CO_2$ and 95% relative humidity. Culture medium was changed every two days and cells were subcultured at 90% confluence.

### 2.2.5 Measurement of cell viability and cellular binding/uptake of NPs in non-differentiated caco-2 cells

Caco-2 cells were seeded into 12-well plates at a density of $2x10^5$ per well, and after 48h was treated with coumarin-6 loaded NPs (0.5mg/ml) suspended in PBS and cell culture medium in the ratio 1:1. After 2, 24 and 48h incubation, cells were washed four times with sterile PBS to remove unbound NPs. Washed cells were harvested using trypsin and treated for viability and binding/uptake studies.

For viability studies, washed cells were centrifuged and re-suspended in propidium iodide solution, incubated for 5minutes and analysed by flow cytometry (FACSCanto, Becton Dickinson, San Jose, CA, USA). Fixed and permeabilised cells were used as positive control and non-treated cells as negative controls (O'Donnell et al., 2015).

For quantitative evaluation of NP binding/uptake, cells were fixed and analysed using flow cytometry (FACSCanto, Becton Dickinson, San Jose, CA, USA) (Kirby et al., 2013).

For evaluation of NP binding/uptake, cells were fixed, their nuclei stained with Hoechst, F-actin was stained with Phalloidin-TRITC, and mounted on the slide using DAKO® Fluorescent Mounting Medium. Images were acquired under the Zeiss confocal laser-scanning microscope (CLSM) (Carl Zeiss, Jena, Germany), Z-sectioning was carried out to visualise NP uptake and transport. Results from the top, middle and bottom stacks are presented (Mattu et al., 2013).

### 2.2.6 Evaluation of cellular uptake, permeability and anti-inflammatory activity of INF-NPs across inflamed Caco-2 cell monolayer

An in-vitro epithelial cell monolayer was developed by culturing Caco-2 cellss continuously over 21 days on microporous polycarbonate membrane filter supports of

0.4 µm pore size in inserts of the Transwell® plate (Costar, Cambridge, MA, USA). Caco-2 cells were seeded at a density of $3x10^5$ cells/well and medium changed every alternate day.

Inflamed intestinal model was obtained by exposing the Caco-2 monolayers at day 21 to the inflammatory mediator, TNF-α (15ng/mL), added to the basolateral chamber and incubated for 48h. Post 48h, the medium in the basolateral chamber was replaced by a fresh culture medium, and NPs/INF-NPs or INF at a concentration of 0.5mg/ml were added to the apical chamber of the healthy and inflamed monolayers and cells were incubated for 48h. Changes in the integrity of the epithelial barrier were determined by measuring the transepithelial electrical resistance (TEER) using EVOM voltohmmeter with chopstick-type electrodes (World Precision Instruments, UK). The basolateral chamber was sampled and the permeability of the fluorescently labelled NPs measured using a multi plate reader (Varioskan Flash, Thermo scientific, Waltham, MA, USA). Cellular uptake of NPs was determined quantitatively by flow cytometry and qualitatively by CLSM. Basolateral chamber was sampled (150uL) and levels of the anti-inflammatory cytokines; IL-8, before addition of INF and INF-NPs and 48 hours post incubation with INF and INF-NPs were measured by ELISA MAX Deluxe (BioLegend, San Diego, CA, USA).

### 2.2.7 Evaluation of cellular binding/uptake and anti-inflammatory properties of INF-NPs in monocytes

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from human blood by density gradient centrifugation on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Monocytes were isolated from PBMCs by negative selection using MACS magnetic separation LS column (Miltenyi Biotec Bergisch Gladbach, Germany)

(Hochart et al., 2006). These monocytes were seeded at 1 x $10^6$ cells per well and treated with 100ng/ml of LPS Escherichia coli 0111:B4 (Invivogen, San Diego, CA, USA) for 36h. Subsequently, the supernatant was replaced by INF solution or INF-NPs at INF dose of 0.5mg/ml suspended in 1:1 PBS and cell culture medium. .After 24h, monocytes were washed and processed, as outlined earlier, for determination of NP binding/uptake by flow cytometry and CLSM. Levels of the anti-inflammatory cytokines; TNF-α and IL-8, were measured by ELISA MAX Deluxe (BioLegend, San Diego, CA, USA).

## 3.0 Results and Discussion

### 3.1 Polymer Characterisation by ATR-FTIR

ATR-FTIR showed that PU, PU-PEG and PCL-PEG polymers were successfully synthesised, as indicated by the signals at 1680 $cm^{-1}$, 3330 $cm^{-1}$ and 1535 $cm^{-1}$ corresponding to the stretching vibration of the carbonyl, the N-H bending and the N-H stretching vibrations of the urethane bond, respectively (Figure not shown). The signals at 1720 $cm^{-1}$ and at 1160 $cm^{-1}$, were attributed to the absorption of the carbonyl and to the C-O stretching of the ester bond in PCL.

A broad signal between 1300-1000 $cm^{-1}$ confirms PEGylation and formation of PU-PEG and PCL-PEG, as the signal at 1110 $cm^{-1}$ is attributed to the C-O-C stretching of the ether bond in PEG.

Molecular weight (Mw) determined by GPC was found to be 42kDa for PU and15 and 25kDafor PCL-PEG and PU-PEG, respectively.

### 3.2 Formulation and Characterisation of NPs

NPs formulated were of average size of 200-283 nm (Table 1), which were reported to be optimal for cell uptake and were also shown to be effective to treat inflammation in IBD. PLGA, PCL and PU NPs showed an average particle size of 200-222nm, a narrow PDI of < 0.2, indicating nearly monomodal distribution and had a zeta potential (ZP) of -13.93 to -15.30 (Table 1). Corresponding PEGylated NPs had a higher PDI of <0.68 and higher ZP values of -4.45 to -6.81. The higher ZP values were related to the shielding effect of the negative charge by PEGylation (Kirby et al., 2013). Morphology of all the NPs was smooth and spherical as shown in figures 1a-f.

**3.3 Influence of polymeric NPs on cell viability**

The viability of caco-2 epithelial cells upon exposure to various NPs for 2, 24 and 48h show no significant change for any of the NPs (ANOVA, post hoc, $p > 0.05$) (Figure 2). This is as expected as the polymers are biodegradable and biocompatible (Danhier et al., 2009; Gentile et al., 2015; Mattu et al., 2013; Rayaprolu and Strom, 2013; Shi et al., 2012).

**3.4 Cellular binding/uptake of Coumarin 6-NPs in non-differentiated epithelial Caco-2 cells**

Cellular interaction, binding and uptake of NPs were dependent on the type of polymeric NPs (Figure 3a & 3b). Cellular interaction and uptake of PLGA and PLGA-PEG NPs was found to be significantly lower and in the range 9-11% (ANOVA, $p < 0.0001$) compared with PCL and PU based NPs and their PEGylated versions. Cellular uptake of PCL and PU NPs were similar, in the range of 65-67% (ANOVA, $p > 0.05$). Corresponding PEG-NPs of PCL and PU showed a significantly higher cellular uptake by 6-12% (ANOVA, $p < 0.05$).

CLSM, Figure 3b, similarly showed a lower intensity and localisation of PLGA and PLGA-PEG NPs indicating lower cellular uptake of these NPs compared to PCL or PU based NPs. PCL and PU based NPs differed in terms of intracellular localisation. PCL and PCL-PEG NPs were found to be scattered in the cytoplasm, and is consistent with the literature (Rayaprolu and Strom, 2013). On the other hand, PU and PU-PEG NPs were located in clusters along the circumference of the nucleus.

NP-cell interaction and binding is a key prerequisite to cellular uptake and internalisation and is defined by NP size and surface properties (Deng et al., 2016; Gaumet et al., 2009; He et al., 2013). In this study, all polymeric NPs were of similar size at ~200nm. The surface charge (ZP) of PLGA, PCL and PU were in the range of -13 to -15mV, while corresponding PEGylated NPs had higher ZP of -4.45 to -6.81. NPs differ in hydrophobicity in the order of PU > PCL > PLGA (Mattu et al., 2013). The higher cellular internalisation of PCL and PU NPs can be related to their higher hydrophobicity, which contributes to their higher cellular interaction and binding, promoting cellular internalisation. A similar trend was reported by Singh et al (Singh et al., 2006) where cellular uptake of PCL NPs in Caco-2 cells was higher compared to PLGA NPs. Mattu et al (Mattu et al., 2013) investigated trastuzumab coated NPs and reported a higher uptake of PU and PCL NPs in breast cancer cells (SKBR3) than PLGA NPs.

The significantly higher cellular uptake of pegylated PU and PCL NPs was related to the near neutral surface charge of these NPs, of -4.45 to -6.85 mV, which promotes cellular interaction, higher binding affinity and internalisation. Do et al (Do et al., 2008) reported that coating of PLGA NPs with PCL-PEG moieties resulted in a significant increase in cellular uptake of PLGA, compared to unmodified PLGA NPs. Similar

results were observed by Mishra et al (Mishra et al., 2004) who reported ~1.4-fold increase in the uptake of PEGylated PEI particles, compared to its unPEGylated form. Based on the results observed, PCL and PU and their corresponding pegylated NPs showed significantly higher cellular uptake than PLGA and PLGA-PEG NPs, did not exhibit any cytotoxicity and were therefore selected for the formulation of INF loaded NPs for investigation in inflamed epithelial cells.

## 3.5 Cell uptake, permeability and anti-inflammatory actitvity of INF-NPs in inflamed Caco-2 epithelial barrier model

### 3.5.1 Characterisation of INF-NPs

Surface adsorption of INF to NPs resulted in a significant increase in NP size (ANOVA, $p < 0.008$) and surface charge (ZP) (ANOVA, $p < 0.05$) for all NPs (Table 1 and 2). The increase in NP size was greater at 3-6 fold compared to an increase in size by ~1.4-fold which we previously reported for the mAb, trastuzumab, on the surface of NPs (Mattu et al., 2013). The increase in size and surface charge of the NPs can be related to the adsorption of INF on the surface of the NPs due to hydrophobic interactions between the hydrophobic surface of the NPs and hydrophobic Fc domain of the mAb, and possibly weaker electrostatic interactions between negatively charged NPs and slightly positively charged INF molecules. The average particle size of INF molecules was 18.46 nm and the ZP was 0.10 at pH 7. At the high concentration of INF (5mg/ml) used, it is possible that in addition to surface adsorption, self association between INF molecules through hydrophobic interaction and bridge formation between adjoining INF-NPs occur, resulting in the large increase in size observed. Adsorption to various surfaces and self-association are characteristics of proteins and IgG molecules, often leading to reversible or irreversible aggregation (Webster, 2013). In this study, the

adsorption was carried out at low temperatures of 4ºC and the INF-NPs lyophilised for stability and storage.

INF loading of the NPs was found to be in the range 48.19- 52.5% w/w and INF bioactivity was not affected by the formulation process (Table 2). The retention of bioactivity confirms that any adsorption and association between drug-drug and drug-NP was reversible.

### 3.5.2 Cell uptake and permeability of NPs across an in-vitro Healthy and Inflamed epithelial cell barrier model

In healthy epithelial barrier model, PU and PU-PEG NPs showed significantly higher uptake compared to PCL and PCL-PEG NPs (Figure 4a) (ANOVA, posthoc, $p < 0.05$). PU and PU-PEG showed ~50% higher permeability at 1.69 and 3.69% compared to PCL and PCL-PEG NPs, which showed a permeability of 0.86 and 2.05%, respectively (Figure 4b). This could be due to higher hydrophobicity of PU compared to PCL NPs. PEGylated NPs showed significantly higher average cellular uptake compared to corresponding non-PEGylated NPs (Figure 4a & b) (ANOVA, posthoc, $p < 0.05$).

Similarly in inflamed epithelial model, INF-PU and INF-PU-PEG NPs showed significantly higher cellular uptake and permeability (ANOVA, posthoc, $p<0.0001$) compared to INF-PCL and INF-PCL-PEG NPs (Figure 4a & b). INF-NPs of PCL-PEG and PU-PEG showed significantly higher uptake (ANOVA, posthoc, $p <0.0001$) and permeability (ANOVA, posthoc, $p <0.05$) than corresponding non-PEGylated NPs. These observations were consistent with analysis by confocal laser scanning microscopy (CLSM) (Figure 5a & b).

NP permeability was higher across the inflamed epithelial barrier model compared to the healthy epithelial barrier (Figure 4b). This was related to the impaired and leaky

tight junction barrier due to inflammation, as shown by the decrease in TEER and is associated with increase in the transepithelial permeability (Maher et al., 2009). Leonard et al (Leonard et al., 2010) observed a 2-fold increase in the apparent permeability of the fluorescein dye across inflamed caco-2 monolayers, compared to the healthy control.

Interestingly, cellular uptake in proliferating non-differentiated caco-2 cells cultured in 12-well non-transwell plates was similar for both PU and PCL NPs (Figure 3a). Whereas, in polarised and differentiated caco-2 monolayers, cultured in Transwells for 21-days, PU NPs showed higher uptake than PCL NPs in both, healthy and inflamed models (Figure 4a). PEGylated forms of PCL and PU showed higher cellular uptake in both, non-differentiated, proliferating Caco-2 cells, as well as in differentiated healthy Caco-2 monolayers (ANOVA, posthoc, $p < 0.0001$), and inflamed cell monolayers (ANOVA, posthoc, $p < 0.002$).

Overall, across all the NP formulations except for PU-PEG NPs, uptake of NPs in polarised and differentiated cellular epithelial barrier model was found to be lower (Figure 4a) than in non-polarised and non-differentiated proliferating caco-2 cells (Figure 3a). This could be related to the difference in orientation of the cells after 21-day culture, whereby caco-2 undergo polarization and enterocytic differentiation into “gut-like” cells that possess microvilli, a basophilic nucleus, junctional complexes, and a tall columnar appearance thus forming an epithelial barrier model (Cartiera et al., 2009).

### 3.5.3 Anti-inflammatory activity of INF-NPs on inflamed epithelial cell barrier model and inflamed monocytes

In this study a decrease in the TEER of caco-2 epithelial barrier model to 65-73% was observed after exposure of the cells to the pro-inflammatory cytokine, TNF-α for 48h (Figure 6a and b). ELISA of the supernanatent cell culture medium showed a 10-fold increase in the secretion of the inflammatory cytokine, IL-8, from 2pg/ml in healthy monolayer to 20pg/ml in inflamed monolayer, similar to that reported by Susewind et al (Susewind et al., 2015).

No change in the TEER was observed when healthy caco-2 epithelial barrier was exposed to the NPs for 48h (Figure 6c and d). CLSM showed distinct F-actin (Figure 5b) confirming that the NPs are not cytotoxic.

Treatment of the inflamed epithelial barrier with INF-NPs resulted in complete TEER and barrier function recovery over 24-48 hours compared to INF treated monolayers and untreated control monolayers (ANOVA, posthoc, $p < 0.05$). The rate and extent of the recovery was dependent on the type of INF-NPs. A significantly higher rate and extent of TEER recovery was observed for INF-PU and INF-PU-PEG NPs compared to INF-PCL and INF-PCL-PEG NPs. Complete TEER recovery at 24hours was observed for INF-PU and INF-PU-PEG NPs. TEER recovery was slowest for monolayers exposed to INF solution (85% at 24h) which was similar to untreated monolayers (82% at 24h,) ($p > 0.05$). IL-8 level after 48 hours treatment with INF and INF-NPs dropped to below level of detection.

The rapid recovery of barrier function observed with PU and PU-PEG NPs can be related to higher cellular uptake and permeability of these formulations across inflamed epithelial barrier model (Figure 4 a&b). Although PEGylated NPs showed higher uptake and permeability (Figure 4 a&b), this was not reflected in the rate and/or

extent of recovery in the TEER, which was not significantly different (ANOVA, posthoc, $p > 0.05$).

CLSM of monolayers after 48h exposure to INF-NPs or INF show that INF-NPs restored the barrier function as evident by distinct and clear F-actin staining, compared to the monolayers treated with INF or untreated monolayes (Figure 5b). Tight junction protein such as occludin and zonula occludens-1 are upregulated during enterocytic differentiation occurring in the continuous 21-day culture of Caco-2 cells on microporous inserts, and these tight junction proteins interact directly with the F-actin in-vitro. Consequently, altered F-actin represents perturbation of tight junction complexes, and thus indicating epithelial barrier disruption.

Anti-inflammatory therapeutics have been reported to result in the recovery of the TEER in the in-vitro cell model of inflammation. Satsu et al reported that anti TNF-α mAb down-regulated inflammation in the Caco-2/THP1 co-culture model in a dose-dependent manner whereby a dose of 1.6μg/ml resulted in a 4-fold recovery in the TEER. On the other hand, Susewind et al (Susewind et al., 2015) reported that 3D co-culture model of Caco-2/macrophages and dendritic cells, treated with IL-1β resulted in a 25% drop in the TEER in 48h, whereas removal of IL-1β reversed the inflammation, and resulted in a recovery in the TEER in 3-4 days.

In this study, the higher rate of recovery in TEER as demonstrated by INF-NPs compared to INF solution was related to the enhanced cellular uptake and permeability of INF-NPs. A similar trend is reported in the literature. Treatment of inflammation induced in 3D co-culture of Caco-2 cells, with free budesonide or its PLGA NPs, showed recovery of TEER and reduction in IL-8 in 24 hours (Leonard et al., 2012a).

PLGA/Eudragit S100 NPs containing curcumin showed a 2.5-fold higher permeability across caco-2 epithelial barrier than curcumin suspension and resulted in a 3-fold

higher anti-inflammatory effects and therapeutic efficacy in in-vitro inflamed macrophages and in-vivo DSS-induced colitis model (Beloqui et al., 2014). Exposure of LPS treated caco-2-BBE monolayers to alginate/chitosan NPs containing an anti-inflammatory tripeptide, for 72h, were reported to result in a dose dependent decrease in the IL8 mRNA expression, whereas the tripeptide solution showed only marginal change in the IL8 mRNA expression (Laroui et al., 2010).

#### 3.5.3.1 Anti-inflammatory activity of INF-NPs in inflamed monocytes

In IBD defects in the intestinal epithelial barrier function causes dysregulation of the underlying mucosal immune cells and release of pro-inflammatory cytokines which results in disease progression and mucosal damage (Neurath, 2014). Neutralising the pro-inflammatory cytokines at source is a key strategy at treating inflammation of the underlying mucosal immune cells. This will alleviate disease progression and promote mucosal healing (Colombel et al., 2014). Novel biodegradable NPs designed in this study have demonstrated higher interaction with inflamed epithelial monolayer resulting in increased cellular uptake and permeability across the monolayer to deliver anti-TNF antibodies. To demonstrate the efficacy of the INF-NPs developed in this work, to neutralise inflammation of immune cells, the INF-NPs were studied in blood derived monocytes stimulated with LPS.

Uptake of INF-NPs by LPS treated inflamed monocytes was found to be higher for PU based NPs and was in the order of PU-PEG>PU>PCL-PEG>PCL (Figure 7a & b). LPS treated monocytes showed a significant decrease in viability of the monocytes (Figure 8a), related to the secretion of inflammatory cytokines, TNF-α and IL-8 (Figure 8b). Treatment of inflamed monocytes with INF or INF-NPs showed an increase in cell

viability resulting from the corresponding decrease in the secretion of TNF-α and IL-8 (ANOVA, posthoc, $p < 0.05$) (Figure 8a & b). INF is known to exhibit high affinity to both, soluble and transmembrane forms of TNF-α (Neurath, 2014). CLSM of LPS treated inflamed monocytes showed blurry F-actin, whereas inflamed monocytes exposed to INF or INF-NPs showed distinct F-actin staining (Figure 7b).

The results indicate that the NPs, in particular PU-PEG NPs, have the potential to act as carriers to deliver their INF cargo to the underlying mucosal cells and neutralise inflammatory cytokines locally, as a result of their ability to translocate across the inflamed intestinal epithelium.

## 4.0 Conclusions

INF loaded NPs of the novel biomaterials of PU and PU-PEG NPs showed highest cellular interaction, uptake and permeability across inflamed epithelial Caco-2 cell monolayer, resulting in rapid recovery of epithelial barrier function (TEER) and reduction of IL-8 and inflammation. The NPs also demonstrated anti-inflammatory properties in blood derived LPS treated monocytes. Their enhanced cellular interaction and translocation across the inflamed epithelial cells demonstrate their potential to cargo anti-TNF-α antibodies such as INF directly to the underlying mucosal immune cells, to neutralise the TNF-α produced and limit inflammation and disease progression. These nanoformulations can potentially be filled in gastroresistant capsules for delivery to the colon where these are reported to be more stable (Yadav et al 2016b). The results obtained in this study may offer an alternative and non-invasive strategy to deliver anti-TNF-α therapeutics to treat gastrointestinal inflammation and warrants further investigation.

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**Declarations of interest:** none

## 5.0 Reference

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Table 1 Characteristics of nanoparticles (NPs)

| **Polymer** | **Molecular weight** | **Concentration (mg/ml)** | **Tween (% w/v)** | **Size (nm)** | **PDI** | **Zeta potential (mV)** |
|---|---|---|---|---|---|---|
| PLGA | 46-50 kDa | 10mg/ml | 2 | 200.43 ± 3.38 | 0.20 ± 0.02 | -14.00 ± 0.46 |
| PCL | 48-90 kDa | 10mg/ml | 2 | 210.93 ± 2.16 | 0.09 ± 0.01 | -13.93 ± 0.12 |
| PU | 42 kDa | 10mg/ml | 2 | 222.75 ± 6.28 | 0.16 ± 0.02 | -15.30 ± 0.37 |
| PLGA-PEG | 52.5 kDa | 55mg/ml | 2 | 287.00 ± 76.08 | 0.68 ± 0.31 | -4.45 ± 5.47 |
| PCL-PEG | 15 kDa | 20mg/ml | 0.02 | 233.65 ± 18.15 | 0.60 ± 0.03 | -4.99 ± 0.12 |
| PU-PEG | 25 kDa | 20mg/ml | 0.02 | 231.55 ± 49.72 | 0.47 ± 0.20 | -6.81 ± 2.67 |

Table 2 Characteristics of infliximab-nanoparticles (INF-NPs)

| Polymer | Size (nm) | PDI | Zeta potential (mV) | INF loading (%) | INF Bioactivity (%) |
|---|---|---|---|---|---|
| INF | 18.46 ± 3.73 | 0.43 ± 0.06 | 0.10 ± 0.17 | - | 101.06 ± 2.94 |
| PCL-INF | 731.37 ± 19.45 | 0.47 ± 0.07 | 0.99 ± 0.65 | 52.50 ± 2.70 | 107.74 ± 0.21 |
| PCL-PEG-INF | 769.13 ± 60.98 | 0.53 ± 0.01 | 1.61 ± 0.15 | 49.76 ± 1.50 | 98.97 ± 3.86 |
| PU-INF | 1537.33 ±120.71 | 0.40 ± 0.07 | -2.52 ± 0.30 | 48.19 ± 5.10 | 100.39 ± 3.07 |
| PU-PEG-INF | 1706.50 ±659.73 | 0.45 ± 0.28 | -2.03 ± 0.25 | 51.82 ± 2.69 | 101.00 ± 3.14 |