Polymeric nanoparticles that combine dexamethasone and naproxen for the synergistic inhibition of *Il12b* transcription in macrophages

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Abstract

Recent studies have demonstrated *in vivo* synergistic immunosuppressive and anti-inflammatory capacity of dexamethasone (Dx) and naproxen (NAP) in collagen-induced arthritis (CIA) rats. However, the molecular basis of this synergistic effect is barely understood. The low solubility of these drugs and their adverse effects hamper their efficacy on the treatment of inflammatory processes making nanoparticulated systems promising candidates to overcome these drawbacks. The aim of this work is the preparation of polymeric nanoparticles (NPs) that combine NAP and Dx in different concentrations, and the evaluation of the expression of key genes related to autoimmune diseases like CIA. To do so, self-assembled polymeric NPs that incorporate covalently-linked NAP and physically entrapped Dx are designed to have hydrodynamic properties that, according to bibliography, may improve retention and co-localization of both drugs at inflammation sites. The rapid uptake of NPs by macrophages is demonstrated using coumarine-6-loaded NPs. Dx is efficiently encapsulated and *in vitro* biological studies demonstrate that the Dx-loaded NAP-bearing NPs are non-cytotoxic and reduce lipopolysaccharide-induced NO released levels at any of the tested concentrations. Moreover, at the molecular level, a significant synergistic reduction of *Il12b* transcript gene expression when combining Dx and NAP is demonstrated.

1. Introduction

A recent study demonstrated the additive immunosuppressive and anti-inflammatory effect of dexamethasone (Dx) and naproxen (NAP) combined treatment with *in vitro* and *in vivo* studies on paw edema in female and male Lewis rats with collagen-induced arthritis (CIA).^[1] Through mathematical models and model simulations authors demonstrated a promising steroid-sparing potential of NAP in CIA rats. However, to better understand these results, authors emphasize on the need of studying the effect of Dx/NAP combinations at a molecular level. CIA is a murine model of rheumatoid arthritis (RA), an autoimmune/inflammatory disorder, in which T cells are the primary dysfunctional cells.^[2] Interleukin-12 (IL12) and interleukin-23 (IL23) are secreted by macrophages and dendritic cells in response to antigen presentation to naïve T cells playing a key role in the differentiation and maintenance of autoreactive T cells.^[3] These ILs are dimeric cytokines sharing IL12-p40 subunit which is encoded by *Il12b* gene.^[4] Actual biological treatments based on monoclonal antibodies blocking IL12-p40 subunit (e.g. ustekinumab) have demonstrated promising results and low side effects if compared with traditional tumor necrosis factor-alpha (TNF- α) inhibitors.^[3] NAP is a well-known non-steroidal anti-inflammatory drug (NSAID) with anti-inflammatory, antipyretic, and analgesic properties. It is associated to the lowest cardiovascular risk of all NSAIDs^[5] and its half-life is more than ten times higher than that of other NSAIDs.^[6, 7] NAP derivatives have recently demonstrated capacity to inhibit IL12 production.^[8] However, NAP is not a disease modifying drug when used alone, thus it is frequently combined with other drugs for the treatment of inflammatory processes. Dx is one of the most frequently used therapeutic glucocorticoids. Owing to its immunosuppressive, and anti-inflammatory capacity, it has demonstrated great potential for the treatment of RA,^[1] Alzheimer,^[9] or inflammatory Bowel disease.^[10] At low doses (i.e. below 100nM) Dx treatment leads to polarization of *in vitro* cultured macrophages to M₂ anti-inflammatory phenotype^[11, 12] whereas at higher doses Dx (approx. 1 μ M) treatment leads to trans-repression of genes encoding pro-inflammatory cytokines like IL12-p40 subunit^[13] and reduces vascular endothelial growth factor (VEGF) expression,^[14-16] a major contributor to macrophages-derived angiogenic activity.^[17] Both drugs are first-line therapies for inflammation-related diseases because of their rapid symptomatic effects.^[5, 18] However, their sustained use is limited by their high hydrophobicity, the rapid clearance of free drug from inflammation sites by the lymphatic system and the dose and time-dependent adverse effects.^[5, 18, 19] It has been well-established the enhanced therapeutic potential of nanomedicines over freely administered drugs. If they are properly designed, they preferentially accumulate in the pathological tissue providing a

better spatial and temporal localization of the drug, and increasing its activity while reducing its adverse effects. ^[20] Features like leaky vasculature or acidic *pH* characterize the inflamed microenvironment when compared with healthy tissue and they can be exploited to favor drug accumulation at inflamed areas. ^[19] Matsumura et al. described for the first time that long-circulating macromolecules undergo passive accumulation in the inflamed tissue as they do in tumor sites through a mechanism similar to Enhanced Permeability and Retention (EPR) effect known as Enhanced Leaky Vasculature and Inflammatory cells Sequestration (ELVIS) effect. ^[21] This name owes to the leaky vasculature that allows preferential accumulation at inflamed areas and the need of sequestration by inflammatory cells to avoid rapid clearance of the macromolecules or nanocarriers by lymphatic drainage which is not present in tumors. Consequently, retention at inflamed tissue can be improved by tailoring nanoparticles (NPs) hydrodynamic properties (e.g. size and surface charge). Cheng et al. ^[22] reported that NPs with diameter below 100 nm are easily cleared by lymphatic vessels and Awaad et al. ^[23] concluded that 95-200 nm is the ideal size for increased cellular uptake. Moreover, NPs presenting positive surface charge showed higher cellular uptake when compared to neutral or anionic particles due to the ionic attraction between cationic charges and negatively charged phospholipids that composes the cell membrane. ^[24, 25] Therefore, the aim of this work was the preparation of polymeric NPs that combine NAP and Dx in different concentrations, and the evaluation of the expression of key genes related to autoimmune diseases like CIA. Self-assembled polymeric NPs that incorporate covalently-linked NAP and physically entrapped Dx were obtained with amphiphilic gradient copolymers presenting appropriate microstructure and hydrophilic/hydrophobic balance to self-assemble in aqueous media. Hydrodynamic properties of the NPs (i.e. size, polydispersity and, surface charge) were optimized as a function of *pH*, NPs final concentration ($[NPs]_F$), and final volume (V_F) to favor retention and co-localization of both drugs at inflammation sites. Stability studies over time were also carried out. Dx was encapsulated and the encapsulation efficacy and loading capacity were determined by HPLC. Finally, RAW264.7 murine macrophages were used as model inflammatory cells. With no further stimuli, they are inactivated macrophages with predominant M_0 phenotype. Activation with pro-inflammatory molecules such as lipopolysaccharide (LPS) leads to polarization to M_1 and overproduction of NO, TNF- α , and, in particular, IL23, and IL12, which are associated to the pathogenesis of autoimmune diseases like CIA. On the contrary, activation with molecules such as Dx polarize macrophages to M_2 phenotype ^[11] leading to overproduction of VEGF, interleukin-10 (IL10) or tumor growth factor-beta (TGF- β) (**scheme 1**). ^[26] According to that, *in vitro* biological

studies with RAW264.7 macrophages were carried out to evaluate rapid uptake of NPs, cytotoxicity and anti-inflammatory capacity of the system. Anti-inflammatory properties were evaluated via NO-released levels quantification using Griess reagent and levels of expression of *Il12b*, *Il23a* and *Tnfa* transcripts by RT-qPCR (**Scheme 1**). The transcript expression levels were assessed under non-activated normal tissue conditions to discard that the positively charged NPs could induce M₁-like polarization through toll-like receptor 4 signaling causing toxicity by interacting with cellular components.^[26, 27] Additionally, levels of expression of M₂ and angiogenesis markers were evaluated after treatment with Dx-loaded systems. And finally, the expression levels of M₁ markers were assessed under simulated inflammatory tissue conditions (*i.e.* after LPS activation of macrophages).

Scheme 1: schematic presentation of the polarization of murine macrophages in response to lipopolysaccharide (LPS) or dexamethasone (Dx) activation and the relation of M₁- and M₂-marker genes under study in this work.

2. Results and Discussion

2.1 Synthesis and characterization of the methacrylic derivative of naproxen (HNAP)

A methacrylic derivative of NAP (HNAP) was successfully synthesized (**Scheme 1a**) in mild conditions with yields above 90% and high purity after a simple purification procedure as confirmed by ¹H-NMR spectroscopy (**figure 1**). The linkage of NAP through the carboxylic group might improve safety profile as it has been reported as the main contributor to gastrointestinal adverse effects.^[28] The resultant ester linkage is prone to hydrolysis under acidic conditions encountered in inflamed tissues where the *pH* is one point lower than physiological *pH* (*i.e.* about 6.4)^[21] and in lumen of lysosomes whenever sequestration by

inflammatory cells occurs.^[29] Altogether may provide a *pH*-accelerated release of NAP at the inflamed area.

Figure 1. ^1H -NMR spectrum of the product of the naproxen-based monomer synthesis. **HNAP:** ^1H NMR (400 MHz, CDCl_3) δ_{H} 7.66 – 7.51 (m, 3H), 7.32 (dd, $J = 8.5, 1.9$ Hz, 1H), 7.12 – 6.98 (m, 2H), 5.87 (dq, $J = 1.9, 1.0$ Hz, 1H), 5.37 (p, $J = 1.6$ Hz, 1H), 4.32 – 4.13 (m, 4H), 3.84 (s, 4H), 1.74 (dd, $J = 1.6, 1.0$ Hz, 3H), 1.51 (d, $J = 7.2$ Hz, 3H).

2.2 Synthesis and characterization of copolymers of naproxen-based methacrylic monomer and 1-vinyl imidazole, poly(HNAP-co-VI).

Previous works in our group have demonstrated that an amphiphilic gradient microstructure is obtained when copolymerizing low reactive vinylic monomers (*e.g.* vinyl-imidazole (VI)) and highly reactive methacrylic ones (*e.g.* HNAP) by free radical copolymerization.^[30-32] Moreover, if the hydrophobic-hydrophilic balance is the appropriate one this microstructure may self-assemble into NPs in aqueous media having a hydrophobic core mainly formed by covalently linked NAP and a hydrophilic shell mainly formed by VI.^[31] As VI also presents protonable amine groups it may provide the desired positive surface charge to favor internalization by inflammatory cells. Accordingly, macromolecules with two different molecular weights and 0.2, 0.5 and 0.8 feed molar composition of HNAP were prepared in order to determine the most appropriate molecular weight and hydrophobic-hydrophilic balance. The obtained copolymers are labeled according to their molecular weight (HMW or LMW) followed by their molar content in HNAP obtained by ^1H -NMR. The chemical reactions carried out for the synthesis of HNAP a) and the copolymers b) are illustrated in **Scheme 2**.

Scheme 2. Synthesis of **a)** the hydrophobic methacrylic derivative of naproxen (HNAP) through a carbodiimide mediated Steglich esterification reaction and; **b)** the copolymer poly(HNAP-*co*-VI) via free radical polymerization reaction in solution.

HNAP was successfully co-polymerized with VI by free radical polymerization for all feed molar ratios as confirmed by the disappearance of the ^1H -NMR signals corresponding to the vinyl protons of both monomers ($\text{CH}_{2\text{-VI}}$ at 4.9 and 5.45 ppm and $\text{CH}_{2\text{-HNAP}}$ at 5.6 and 5.9 ppm), the new ^1H -NMR signals resultant from the methylene protons of the backbone chains ($\text{CH}_{3\text{-m}}$, $\text{CH}_{2\text{-l}}$ and $\text{CH}_{2\text{-n}}$ between 0.1 and 2.8 ppm) and the broadening of the signals as a result of the macromolecular nature of the copolymer (**figure 2**). The copolymers molar composition was quantitatively determined from their corresponding ^1H -NMR spectra by considering the signals between 0.1 and 2.8 ppm assigned to 8 protons of HNAP ($\text{CH}_{3\text{-m}}$, $\text{CH}_{3\text{-i}}$ and $\text{CH}_{2\text{-l}}$) and 2 protons of VI ($\text{CH}_{2\text{-n}}$) and the signals between 6.5 and 8.0 ppm resultant from 6 aromatic protons of HNAP ($\text{CH}_{\text{b-g}}$) and 3 aromatic protons of VI ($\text{CH}_{\text{p,q,r}}$).

Figure 2. ^1H -NMR spectrum of HNAP71 copolymer and the integrals used to compute copolymer molar composition. *p*(HNAP-co-VI) (71-29; HNAP-71): ^1H NMR (400 MHz, DMSO_{d6}) δ_{H} 7.89–6.45 (m, 9H (3VI + 6HNAP)), 4.53–3.39 (m, 8H (8HNAP)), 3.22–2.73 (s, 1H (1VI)), 2.28–0.10 (m, 10H (2VI + 8HNAP))

HNAP feed molar composition (F_{HNAP}), homopolymer and copolymer HNAP molar composition (f_{HNAP}), reaction yields, average molecular weight (M_w), polydispersity index ($\mathcal{D}$) and glass transition temperature (T_g) of the synthesized copolymers are summarized in **table 1**.

Table 1. Summary of the main results of the physicochemical characterization of high and low molecular weight copolymers and homopolymers.

	Polymer	$F_{\text{HNAP}}^{\text{a)}$	$f_{\text{HNAP}}^{\text{b)}$	Yield [%]	$M_w^{\text{c)}$ [kDa]	$\mathcal{D}^{\text{d)}$	$T_g^{\text{e)}$ [°C]
HMW	H-pVI	0.00	0.00	22	26	1.3	177
	H-HNAP26	0.20	0.26	43	164	1.6	82
	H-HNAP60	0.50	0.60	76	249	1.8	75
	H-HNAP84	0.80	0.84	96	331	1.9	58
	H-pHNAP	1.00	1.00	94	343	2.0	56
LMW	L-pVI	0.00	0.00	33	33	1.6	184
	L-HNAP24	0.20	0.24	72	62	1.9	84
	L-HNAP71	0.50	0.71	75	75	2.0	67
	L-HNAP85	0.80	0.85	77	83	1.9	58
	L-pHNAP	1.00	1.00	84	96	2.3	57

^{a)}Molar fraction of HNAP in the feed, ^{b)}molar fraction of HNAP in the copolymer, ^{c)}Molecular weight, ^{d)}Polidispersity of molecular weight distribution and ^{e)}glass transition temperature

The polymerization successfully occurred in the whole range of compositions and with both initial monomer concentrations tested. Copolymers with molecular weights below 100 KDa were obtained when using a monomer initial concentration of 0.25 M and copolymers with molecular weights above this value resulted when using twice this initial monomer concentration as expected for a free radical copolymerization reaction.^[33] The differences among copolymer HNAP molar content (f_{HNAP}) and feed HNAP molar content (F_{HNAP}) can be attributed to the differences on the chemical reactivity of the monomers (see next section), and because total conversion of the polymerization was not reached. Moreover, it has been reported that copolymerization at high concentrations of VI are accompanied by a mild degradative addition reaction that depends on the interaction of the VI ring with the propagating radicals.^[34] These relative degradative addition reactions may explain the low yields and low polydispersity indexes obtained for copolymers with higher F_{VI} and the increasing trend of molecular weights as F_{HNAP} increases being this effect more significant in high monomer concentration reactions (*i.e.* HMW). Polydispersity index values and yields approaches to the typical ones for a conventional radical polymerization process as F_{HNAP} increases. According to the obtained reactivity ratios (see next section) a pseudoblock microstructure was obtained, however only one T_g between the range of the T_g values of both homopolymers (*i.e.* pHNAP and pVI) was detected for all copolymers. This result indicates that microphase segregation was not produced or was not detected by the applied DSC method.

2.3 Determination of Reactivity Ratios: Microstructural Analysis

Reactivity ratios are kinetic parameters which measure the relative rate of addition of each comonomer to a growing copolymer chain and they allow the estimation of the copolymer average composition and the monomer sequence distribution. Those comonomers that differ in hydrophilicity and present very different reactivity ratios give rise to amphiphilic gradient microstructure by free radical copolymerization.^[30, 32] Accordingly, the most reactive one is incorporated first and the least reactive one is incorporated at the last stages of the copolymerization reaction. These copolymers self-assemble in aqueous media leading to supramolecular structures (*e.g.* NPs). Reactivity ratios of the co-monomers (r_{HNAP} and r_{VI}) were determined by in situ ¹H-NMR monitorization of the copolymerization reaction at three different feed molar compositions by following the disappearance of the vinyl protons signals

of both co-monomers, as previously described by Aguilar et al.^[35] The data obtained were fit to a well-known integrated form of the differential copolymer equation defining the terminal model:

$$[HNAP]_t = [HNAP]_o \times \left(\frac{[VI]_t / [HNAP]_t}{[VI]_o / [HNAP]_o} \right)^{\frac{r_{VI}}{1-r_{VI}}} \times \left(\frac{1 - r_{VI} + (r_{HNAP} - 1)[VI]_t}{1 - r_{VI} + (r_{HNAP} - 1)[VI]_o} \right)^{\frac{(r_{HNAP}r_{VI})-1}{(1-r_{HNAP})(1-r_{VI})}}$$

Being $[M]_t$ and $[M]_o$ the molar concentration of monomer M at time t and the initial monomer concentration, respectively; and r_M the reactivity ratio of monomer M . The obtained reactivity ratios were $r_{HNAP} = 3.16 \pm 1.2$ and $r_{VI} = 0.31 \pm 0.06$ meaning that active radicals incorporate HNAP monomer around 10 times faster than VI. These values were consistent with previously reported copolymerization reactions between VI and methacrylic derivatives using AIBN as free radical initiator^[34, 36] and they revealed that the desired gradient microstructure is obtained. **Figure S1** shows the surface copolymerization diagram representing the instantaneous HNAP incorporation to the growing chain as a function of conversion and HNAP feed molar composition. Macromolecules rich in the most reactive monomer (HNAP) are formed at low conversions, and after most HNAP is consumed, VI-rich copolymer chains are formed. Few chains with intermediate compositions are obtained.

2.4 Preparation and characterization of self-assembled polymeric nanoparticles.

NPs were obtained by nanoprecipitation method in aqueous media for all the prepared copolymers. Results of hydrodynamic diameter (D_h , by intensity) and polydispersity index (PdI) obtained by DLS and zeta potential (ξ) values obtained by LDE are summarized in **table 2**.

Table 2. Hydrodynamic properties of nanoparticles based on HNAP26, HNAP60, HNAP84, and HNAP24, HNAP71, and HNAP85 measured by Dynamic Light Scattering and Laser Doppler Electrophoresis.

	Copolymer	D_h^a [nm]	PdI^b	ξ^c [mV]
HMW	NP-H-HNAP26	140.3 $\pm$ 7.8	0.358 $\pm$ 0.058	+ 23.8 $\pm$ 1.8
	NP-H-HNAP60	180.3 $\pm$ 3.5	0.366 $\pm$ 0.058	+ 32.2 $\pm$ 0.9
	NP-H-HNAP84	231.5 $\pm$ 9.2	0.498 $\pm$ 0.084	+ 39.3 $\pm$ 1.0
LMW	NP-L-HNAP24	90.6 $\pm$ 1.3	0.175 $\pm$ 0.015	+ 25.3 $\pm$ 0.8
	NP-L-HNAP71	140.2 $\pm$ 2.1	0.181 $\pm$ 0.002	+ 28.8 $\pm$ 0.7

NP-L-HNAP85	191.0 ± 6.8	0.375 ± 0.054	+ 40.6 ± 0.8
^{a)} Hydrodynamic diameter, ^{b)} Polydispersity Index and ^{c)} z-potential			

NPs were labeled as the copolymers that were used in their preparation. High molecular weight copolymers did not lead to the formation of stable NP because of their poor solubility in the organic phase. Both copolymer composition and molecular weight played a key role in the NP stability and polydispersity, being $PdI > 0.3$ for all the NP obtained with HMW copolymers. The formation of stable NPs was only feasible for LMW formulations with f_{HNAP} lower than 0.80. HNAP85-based NPs and NPs based on HMW copolymers presented high polydispersity indexes and precipitation of the copolymer was observed shortly after preparation. VI is located at the surface of the NPs as indicated by the positive surface charge. Moreover, the ζ values around + 30 mV for the HNAP71-based formulation are indicative of good stability in suspension. ^[37] NP-L-HNAP71 showed spherical morphology (**figure 3**), a hydrodynamic diameter of 140 nm (*i.e.* between 100 and 200 nm), and positive surface charge ($\zeta = +28.8$ mV). Therefore NP-L-HNAP71 presented the most suitable hydrodynamic properties for passive accumulation ^[22, 38] and improved retention at inflamed areas ^[24, 25] according to bibliography.

Figure 3. Scanning Electron Microscopy micrograph showing morphology of NP-L-HNAP71.

NP diameter slightly varied (*i.e.* ± 5 -10 nm) with $[NPs]$ on the aqueous phase or with final volume keeping the values within the ranges that facilitate their sequestration by inflammatory cells. However, the particles diameter significantly increased up to 20 nm when the concentration of copolymer in the organic phase changes from 10 mgmL⁻¹ to 15 mgmL⁻¹ (**table 3**).

Table 3. Hydrodynamic properties of nanoparticles based on HNAP71 at different final concentration of nanoparticles in the aqueous phase and at different final volumes.

$[NPs]_{A.P.}^{a)}$ [mgmL ⁻¹]	V_F (mL)	$[HNAP71]_{O.P.}^{b)}$ [mgmL ⁻¹]	$D_h^{c)}$ [nm]	$Pdl^{d)}$	$\xi^{e)}$ [mV]
1.0	10	10	140.2 ± 2.1	0.181 ± 0.002	28.8 ± 0.7
	20	10	126.6 ± 1.4	0.186 ± 0.021	27.8 ± 0.1
	30	10	132.1 ± 1.6	0.173 ± 0.023	30.6 ± 1.4
5.0	10	10	129.5 ± 0.2	0.164 ± 0.025	31.2 ± 1.5
	20	10	131.7 ± 1.3	0.165 ± 0.021	29.3 ± 0.8
	30	15	150.0 ± 2.9	0.197 ± 0.016	28.8 ± 1.2

a) Concentration of NPs in the aqueous phase and *b)* concentration of the copolymer in the organic phase *c)* hydrodynamic diameter, *d)* polydispersity and *e)* zeta potential

VI ionizable groups played a key role in the NP stability as a function of *pH*. **Figure 4a** shows the size distribution and the ζ values of the HNAP71 nanoparticulated system at different *pH*. When *pH* 5.0 is reached, it is observed a broadening of the size distribution and a significant increase in the intensity of the peak in the microscale suggesting the agglomeration of NPs that may be attributed to the decrease in the surface charge and, hence, to the reduction of the electrostatic repulsion between particles as the *pH* approaches to the pK_b of VI ($pK_b = 5.0$ -6.0). The phenomenon is partially reversible as when *pH* was reduced to 4.0 original hydrodynamic properties are recovered. Based on these results and on the previously reported strong buffering capacity of VI, ^[39, 40] it was necessary the use of 0.1 M Acetic Acid to keep *pH* value constant avoiding NPs agglomeration and maintaining their stability over time. **Figure 4b** shows that there are no significant changes on the hydrodynamic properties of HNAP71 NPs obtained at *pH* 4 and stored during 4 weeks at 4 °C. Furthermore, NPs recover their initial hydrodynamic properties after freeze-drying and dispersion in the buffer solution at *pH* 4.0 only if they are sonicated for 10 minutes with an ultrasound tip (30% amplitude) (**figure 4c**). Therefore, size and morphology of the particles could be modulated by controlling the molar composition of the copolymers and the concentration of copolymer in the organic phase and NPs can be stored at 4 °C and *pH* 4.0 for at least one month or in powder.

Figure 4. Size distribution (by intensity) and z-potential (ξ) of NPs based on HNAP71 measured **a)** at different pH values, **b)** at pH 4 and 4 °C for different periods of time and; **c)** before freeze-drying (dashed line) and after freeze-drying and tip sonication for 10 minutes (solid line).

2.5 Dexamethasone loaded NPs

Dx was encapsulated in NP-L-HNAP71 with encapsulation efficacy of 8% and loading capacity of 2% showing a diameter increase of 18 nm which translates into a reduction on the surface area-to-volume ratio and hence, lead to a decrease of the surface charge (**table 4**).

Table 4. Summary of encapsulation efficiency, loading capacity and hydrodynamic properties of unloaded and, dexamethasone-loaded nanoparticles prepared with L-HNAP71 copolymer

Acronym	Sample	EE ^{a)} [%]	LC ^{b)} [%]	D_h ^{c)} [nm]	PdI ^{d)}	ξ ^{e)} [mV]
NAP NPs	NP-L-HNAP71	-	-	131.5 $\pm$ 3.3	0.115 $\pm$ 0.016	+ 28.8 $\pm$ 0.7
8Dx-NAP NPs	8Dx-NP-L-HNAP71	8	2.00	149.5 $\pm$ 1.8	0.085 $\pm$ 0.018	+ 26.1 $\pm$ 0.9

^{a)}Encapsulation Efficiency, ^{b)}Loading capacity, ^{c)}hydrodynamic diameter, ^{d)}polydispersity and ^{e)}zeta potential

2.6 Uptake of c6-labelled NPs by macrophages

Figure 5. a) Fluorescence micrograph of c6-NAP NP internalized by RAW264.7 macrophages; and b) quantification of the internalized c6 by RAW264.7 when treated with c6-NAP NP for different periods of time. The diagram includes the mean and the standard deviation ($n = 3$).

Fluorescent c6-loaded NPs (**table S1**) were used to monitor cellular internalization by RAW264.7 macrophages over 24h of exposure. **Figure 5** shows that the NPs uptake by RAW264.7 macrophages increases linearly over time and fluorescence images confirmed the successful internalization of the NPs when in suspension. These results are in accordance with recently reported fast uptake of NAP-containing NPs by THP1-derived macrophages.^[41] The uptake of nanoparticles by inflammatory cells is critical to retain them in the inflamed tissue and, in this work, hydrodynamic properties were adjusted to enhance the ELVIS effect according to bibliography, *i.e.* hydrodynamic diameters between 100 and 200, positive zeta potential, and spherical shape.^[22-25]

2.7 *In vitro* cytotoxicity assay of NPs

Cytotoxicity of 8Dx-NAP NPs was studied and compared with the free Dx at a concentration equal to the Dx encapsulated in 8Dx-NAP (*i.e.* 56.66 μM of Dx per 1 mgmL⁻¹ of NPs) and NAP NP using RAW264.7. None of the assayed NP or Dx concentrations resulted on a reduction in viability of RAW264.7 higher than 30% (ISO 10993-5:2009) after 24h. 8Dx-NAP NPs (dashed white) and unloaded NAP NPs (black) formulations showed non-

statistically significant differences (* $p < 0.05$) with respect to the control (CP i.e. cells in culture medium, LPS-) at any of the tested concentrations but the highest one (0.25 mgmL^{-1}) in case of NAP NPs and the two highest ones (0.25 and 0.125 mgmL^{-1}) in case of 8Dx-NAP NPs. However, free Dx (red) presented significant differences with the control at all tested concentrations. These results suggest that encapsulated Dx presents lower cytotoxicity than free-Dx (**figure 6**), however, no statistically significant differences were found between these two groups after 24h of incubation.

Figure 6. Cell viability of RAW264.7 macrophages treated with different concentrations of unloaded NPs (NAP NPs, black), Dx-loaded NAP NPs (8Dx-NAP NPs, white) or free Dx (red) over 24h. The diagrams include the mean, the standard deviation ($n = 8$), and the ANOVA results (* $p < 0.05$ statistically significant difference with cells treated with media (LPS-, top of the bars) and between the different systems (brackets): NAP NPs, 8Dx-NAP NPs and free Dx).

2.8 In vitro anti-inflammatory and anti-angiogenic activity of NPs

Nitric Oxide (NO) assay

LPS-induced NO release was measured after 24h of LPS administration and challenged with different concentrations of 8Dx-NAP NPs, unloaded NAP NPs or free Dx (**figure 7**).

Figure 7. NO release by RAW264.7 macrophages after 24 h with no treatment (LPS-), treatment with LPS alone (LPS+), and treatment with LPS and different concentrations of unloaded NAP NPs (black), 8Dx-NAP NPs (white) or free Dx (red) suspension. The diagrams include the mean, the standard deviation ($n = 8$), and the ANOVA results (* $p < 0.05$ and ** $p < 0.01$ statistically significant difference with LPS+ cells (on top of the bars) or between NAP NPs, 8Dx-NAP NPs and free Dx (brackets)).

After 24h of incubation, free Dx and 8Dx-NAP reduced NO release in a statistically significant manner at all tested concentrations whereas unloaded NAP NPs counteract LPS-induced NO release in a statistically significant (* $p < 0.05$) way with respect to LPS treated cells (LPS+) at all concentrations but 0.09 and 0.045 mgmL^{-1} (**figure 7**). At NPs concentration between 0.125 and 0.023 mgmL^{-1} , it is observed a statistically significant (* $p < 0.05$) reduction of NO released levels when comparing unloaded NAP NPs and 8Dx-NAP NPs. Moreover, for this range of concentrations 8Dx-NAP NPs and free Dx present non-significant differences in terms of reduction of % NO-release. These results demonstrate that encapsulation does not diminish Dx capacity to reduce NO-release levels at NPs concentrations between 0.125 and 0.023 mgmL^{-1} . A concentration of NP of 0.045 mgmL^{-1} was chosen for further experiments as it was the lowest non-toxic concentration of NPs in which most significant differences between unloaded NPs and Dx-loaded NPs were observed.

Reverse transcription, real-time quantitative PCR (RT-qPCR) analysis

Figure 8a and figure 8b, respectively, show the fold change in transcript levels of *Tnfa*, *Il12b* and *Il23a* (M_1 markers) and *Vegfa* (angiogenesis and M_2 marker), *Tgfb1* and *Il10* (M_2 markers) determined by RT-qPCR after 1 day and 7 days of treatment. Results are expressed relative to the corresponding level of expression of each transcript in the untreated sample (*i.e.* only culture media, LPS-) and they are the graphical presentation of the mean and standard deviation (mean $\pm$ SD, **table S2**) of two independent samples ($n = 2$). The heat map in **figure 8c** showed that after 24h of treatment with 8Dx-NAP NPs or NAP NPs, all M_1 markers were under-expressed (green) or equally expressed (black) when compared to the untreated sample showing no M_1 -polarization at this time point. In contrast, all genes but *Il12b* were overexpressed (red) after 7 days post treatment with any of the nanoparticulated systems tested. However, the statistical analysis revealed no significant differences in the expression of *Tnfa* or *Il23a* between systems and that only *Tnfa* expression and *Il23a* expression after 7 days of treatment with Dx-loaded NPs and free Dx, respectively, were significantly higher when compared to the untreated sample (LPS-). The *Tnfa* increased level after long-term treatment might be related with the presence of an NSAID in the system, an extensively reported effect related to important gastrointestinal side effects.^[42] Basically, NSAIDs counteract prostraglandin-E2 (PGE2) overproduction and hence, reduce PGE2 positive feedback on TNF- α level.^[43] This effect was not observed after 24 hours of treatment as with other previously described free NSAIDs treatments.^[44] However, after 7 days, *Tnfa* levels increased in an evident and comparable manner for the three systems under study and significant differences with respect to the untreated control (LPS-) were only observed for the system combining NAP/Dx. Therefore, the linkage of the NSAID to the polymeric structure and its controlled release delays the onset of this response whereas Dx encapsulation did not counteract it. In fact, this effect is even increased by NAP/Dx combination because Dx is a glucocorticoid that inhibits phospholipase A2, an upstream mediator in the production of prostaglandins from arachidonic acid, and hence, it can ultimately contribute to a further reduction of PGE2 levels.^[45] All in all, the most interesting result was the almost knockdown of *Il12b* expression when treating RAW264.7 macrophages with 8Dx-NAP NPs. According to the heat map, Dx in its free form inhibited *Il12b* expression levels at both time points tested. NAP NPs also inhibit *Il12b* expression (1 day) or kept it at basal levels (7 days). However, statistically significant differences were only observed after treatment with the Dx/NAP nanoparticulated system. In fact, there was a clear synergistic effect in the trans-repression of this gene when encapsulating Dx in NAP-bearing NPs. It has been reported that *Il12b*^{-/-} mice

are completely protected from CIA. ^[46] Therefore, if this synergistic repression of *Il12b* occurs also under inflammatory conditions, it might be the reason for the Dx dose-sparing capacity of NAP in the treatment of this disease. On the other hand, **figure 8b** shows that, as expected, no statistically significant over-expression of M₂ markers is observed for either free Dx or encapsulated Dx (2.55 μ M) as the concentration of Dx is higher than 100 nM which is the reported limit concentration for polarization. ^[12] At concentrations higher than 100 nM, Dx is expected to reduce *VEGF* expression, a major contributor to macrophage-derived angiogenic activity, ^[17] and also *Il10* expression contributing to reduction of allergic inflammation in the treatment of allergic diseases. ^[47] This anti-angiogenic capacity described for Dx in several cell lines ^[15, 48] is clearly observed after 7 days when it is in its free form (free Dx) but not when encapsulated (8Dx-NAP-NPs). The same occurs with the *Il10* transrepression which was clearly observed in Dx on its free form at both time points studied but was not occurring when cells were treated with 8Dx-NAP-NPs instead. Therefore, encapsulation and controlled release of the drug or its combination with NAP is causing retardation or suppression of anti-angiogenic activity and transrepression of *Il10*. The reduction of *Il10* repression caused by Dx when combining it with NAP might be related to the observed synergistic repression of *Il12* as it has been reported that blockage of IL10 translates into IL12 overproduction.

Figure 8. Quantitative real-time PCR data. Fold change in gene expression levels with respect to control (cells treated with medium, LPS-) of **a)** M₁ markers or **b)** M₂ markers in non-

inflamed samples treated with free dexamethasone (free Dx, 2.55 μM), Dx-loaded naproxen-bearing NPs (8Dx-NAP NPs, 2.55 μM Dx and 0.045 mgmL^{-1} NPs) and unloaded naproxen-bearing NPs (NAP NPs, 0.045 mgmL^{-1}) for 1 day or 7 days. Results are expressed relative to the corresponding level of expression of each transcript in the untreated sample (i.e. only culture media). Data are presented as mean fold changes in gene transcript levels (mean $\pm$ standard deviation) in two independent biological samples per system (three experimental replicates per biological sample), and the ANOVA results (# - comparison between day 1 and day 7 conditions, # $p < 0.05$ and ### $p < 0.001$; * - comparison between systems at the same time point, ** $p < 0.01$ and *** $p < 0.001$).

Finally, as *Il12b* is primarily produced by activated macrophages, to confirm Dx/NAP synergistic effect under simulated inflammatory conditions, macrophages are polarized to M₁ pro-inflammatory phenotype by LPS-stimulation.^[49] At the same time, they are treated with either culture media (LPS+), free Dx (2.55 μM), unloaded NPs (0.045 mgmL^{-1}) or Dx-loaded NPs (2.55 μM Dx and 0.045 mgmL^{-1} NPs). Again, the expression of M₁ marker genes was expressed relative to the corresponding level of expression of each transcript in the untreated sample (**figure 9**). When comparing to the inflamed control (LPS+), after 24 hours of treatment with NAP NPs transcript levels of pro-inflammatory cytokines were not altered. However, free Dx and 8Dx-NAP NPs treatment significantly reverse M₁ polarization. Therefore, the short-term activity of the system is attributed to Dx release. Moreover, the system combining covalently linked NAP and physically entrapped Dx (8Dx-NAP NPs) reduced in a highly significant manner the levels of *Il12b* at both time points reproducing the previously obtained results in normal tissue conditions. The significantly increased expression levels of *Tnfa* that is clearly observed in Dx/NAP combining system might also play a role on *Il12b* synergistic repression. Some authors reported that this increase in *Tnfa* is not necessarily pathogenic but it could be beneficial as it regulates adaptive immune response via inhibition of IL12 and IL23.^[50] These results support this affirmation and suggest that *Tnfa* induced inhibition of IL12 and IL23 occurs through repression of *Il12b* gene, a gene codifying for IL12-p40 subunit.

Figure 9. Quantitative real-time PCR data. Gene transcript levels of M1 markers in inflamed samples (500 ng/mL of LPS) treated with just LPS (LPS+) or LPS and free dexamethasone (free Dx, 2.55 μ M), Dx-loaded naproxen-bearing NPs (8Dx-NAP NPs, 2.55 μ M Dx and 0.045 mgmL⁻¹ NPs) and unloaded naproxen-bearing NPs (NAP NPs, 0.045 mgmL⁻¹) for 1 day or 7 days. Results are expressed relative to the corresponding level of expression of each transcript in the untreated sample (LPS-). Data are presented as mean fold changes in gene transcript levels (mean $\pm$ standard deviation) in two independent biological samples per system (three experimental replicates per biological sample), and the ANOVA results (# - comparison with LPS+ control, #p < 0.05 and ###p < 0.001; * - comparison with untreated LPS- control, *p < 0.05, **p < 0.01 and ***p < 0.001)

The study at the molecular level of the expression of specific markers of autoimmune diseases confirmed that the combination of NAP and Dx translates into a synergistic reduction of *IL12b* transcript levels either in normal or inflammatory conditions. *IL12b* gene transcribes for the IL12-p40 subunit that is common for IL12 and IL23. IL12 is decisive in regulating lineage commitment to T helper 1 cells (Th1) development, whereas IL23 is essential for the maturation and stability of IL17-producing T helper 17 cells (Th17).^[46] Therefore, Dx/NAP combination and, in particular, the newly described Dx-loaded NAP bearing NPs have potential for the treatment of diseases in which IL12 and IL23 are overexpressed and Th1 and Th17 play a key role in pathogenesis. That is the case of autoimmune/inflammatory disorders like Crohn's disease, ulcerative colitis, psoriatic arthritis, rheumatoid arthritis, human multiple sclerosis, psoriasis...^[51, 52] This combination of FDA-approved drugs acts at a genetic level inhibiting *IL12b* expression levels by macrophages, a IL12-p40 subunit codifying gene, and, hence, decreasing IL12 and IL23 levels. This could offer an alternative to an antibody

(Sterala© (ustekinumab)) recently approved by the FDA for the treatment of Crohn's disease, psoriasis, psoriatic arthritis and plaque psoriasis. This monoclonal antibody blocks IL12-p40 subunit preventing IL12 and IL23 binding to their receptors. ^[53, 54]

3. Conclusions

New self-assembled bioactive NAP-containing polymeric NPs loaded with Dx were successfully prepared. Hydrodynamic properties and surface charge were optimized to favor accumulation and retention at inflamed areas. In vitro biological tests with RAW264.7 macrophages demonstrated fast sequestration of c6-loaded NPs. Moreover, the system was non-cytotoxic and Dx encapsulation does not compromise the NO reduction capacity at any of the tested concentrations. Finally, the most interesting result was the synergistic reduction of *Il12b* transcript levels when combining Dx and NAP. A clear Dx dose-sparing capacity of NAP was observed in terms of *Il12b* repression which makes this combination highly interesting in the treatment of autoimmune/inflammatory diseases in which IL12 or IL23 are overexpressed and Th1 and Th17 cells play a key role in pathogenesis. This drug delivery system has been designed, according to bibliography, to have potential to achieve better spatiotemporal localization of Dx/NAP combination at inflamed areas and, by the synergistic reduction of *Il12b* transcript levels, to improve the treatment of a wide range of autoimmune diseases.

4. Experimental Section

Synthesis and characterization of the methacrylic derivative of naproxen (HNAP). The synthesis of the HNAP was carried out through a carbodiimide mediated Steglich esterification reaction ^[55] (Scheme 1) as previously described by our group for the methacrylic derivative of ibuprofen. ^[31] Briefly, NAP (1 equiv) was dissolved in dichloromethane (DCM, Aldrich) and subsequently, purified hydroxyethyl methacrylate (HEMA, Aldrich, 1 equiv) and 4-dimethylaminopyridine (DMAP, Aldrich, 0.1 equiv) were also added to the reaction mixture. Dicyclohexylcarbodiimide (DCC, Fluka, 1 equiv) was then added dropwise under continuous stirring and N₂ (g) atmosphere. The resultant solution was stirred for 24h at room temperature. The reaction product was filtered to eliminate N,N'-dicyclohexylurea formed salt and the organic phase was washed with water and a saturated solution of NaHCO₃ (Panreac). Formerly, the resultant solution was dried with a saturated NaCl (Panreac) solution and anhydrous MgSO₄ (Qemical) and finally, DCM was evaporated under reduced pressure. The

chemical structure and purity of HNAP was elucidated by ^1H -NMR spectroscopy dissolving sample in deuterated chloroform (CDCl_3 , Sigma-Aldrich).

Synthesis and characterization of copolymers of naproxen-based methacrylic monomer and 1-vinyl imidazole, poly(HNAP-co-VI). Copolymeric systems based on HNAP were synthesized by free radical polymerization in solution using 1-vinylimidazole (VI, Aldrich) as co-monomer (**Scheme 1**). Three HNAP:VI feed molar ratios (*i.e.* 20:80, 50:50, 80:20) were tested. Moreover, two initial monomers concentrations were used: 0.5 M and 0.25 M. The copolymers were named HNAP followed by their molar content in this monomer. Particularly, comonomers were dissolved in dimethylsulfoxide (DMSO, Scharlau) at a total monomer concentration of 0.5 M or 0.25 M, and the reaction mixtures were deoxygenated with N_2 (g) for 10 minutes. Then, 2,2'-azobisisobutyronitrile (AIBN, Merck, 1.5×10^{-2} M) was to the solution and the reaction vessel was transferred to an oven at 60 °C for 12 hours. Copolymerization mixtures were purified by dialysis (Spectrum Laboratories, 3.5 KDa molecular weight cut-off) against distilled water for 72 hours. Copolymers were isolated by freeze-drying as a white powder. Copolymers molar composition, molecular weight and polydispersity of molecular weight distribution and, glass transition temperature were determined by ^1H -NMR, SEC and DSC, respectively (**Supplementary Information**).

Determination of reactivity ratios. Reactivity ratios of the comonomers were determined by “in situ” ^1H -NMR monitorization of the copolymerization reaction according to a previously described protocol.^[35] Briefly, copolymerization reactions ($F_{\text{HNAP}} = 0.20, 0.40$ and 0.60 ; $[M] = 0.25$ M and $[AIBN] = 0.015$ M) were carried out inside a NMR tube in the same experimental conditions as described above but using deuterated DMSO (DMSO-d_6 , Merck) as a solvent. A solution of DMF (10 mg mL^{-1}) in DMSO-d_6 in a thin wall capillary tube introduced in the NMR tube was used as reference. Temperature was maintained at 60 °C using the apparatus (Varian Mercury 400 MHz) heat control system. Signals were integrated using MestreNova 9.0 software and reactivity ratios were determined using the methodology described by Aguilar, M.R. et al. in 2002.^[35]

Preparation and characterization of self-assembled nanoparticles. Self-assembled NPs were prepared via nanoprecipitation method. Briefly, copolymers were dissolved in a 8:2 (v:v, acetone:ethanol, Scharlau) solvents mixture at a concentration of 10 mg mL^{-1} and then added dropwise over a continuously stirred aqueous buffer solution at pH 4 (0.1 M Acetic Acid and 100 mM NaCl), a pH below the reported pK_b of VI (*i.e.* 5.5-6.1).^[56, 57] The resultant suspension was stirred overnight to evaporate remaining organic solvent. NPs were stored in suspension at 4 °C. Hydrodynamic properties of NP suspension obtained by nanoprecipitation

of the high molecular weight and the low molecular weight copolymers (HNAP26, HNAP60, HNAP84, and HNAP24, HNAP71, HNAP85, respectively) were measured right after preparation of NPs to optimize hydrophobic-hydrophilic balance for NPs self-assembly in aqueous medium. In order to establish proper preparation conditions of NPs, NP-L-HNAP71 hydrodynamic properties were also measured at different final concentrations of NPs in aqueous suspension ($[NPs]_F = 1 \text{ mgmL}^{-1}$ and 5 mgmL^{-1}) and final volumes of the aqueous phase ($V_F = 10 \text{ mL}$, 20 mL and 30 mL). In addition, to establish proper storage conditions, they were measured at different pH values (4.0, 4.5, 5.0 and, 5.5) and time-points (0 days, 7 days, 14 days, 21 days and, 28 days) (**see supplementary information for detailed explanation**). In particular, the particle size distribution of the NP suspension was determined by dynamic light scattering (DLS) using a Malvern Nanosizer NanoZS Instrument equipped with a 4 mW He-Ne laser ($\lambda = 633 \text{ nm}$) at a scattering angle of 173° . Measurements of NP dispersions were performed in square polystyrene cuvettes (SARSTEDT) and the temperature was kept constant at 25°C . The autocorrelation function was converted in an intensity particle size distribution with ZetaSizer Software 7.10 version, to get the mean hydrodynamic diameter (D_h) and the particle dispersion index (PdI) between 0 (monodisperse particles) and 1 (polydisperse particles) based on the Stokes–Einstein equation, assuming the particles to be spherical. For each sample, the statistical average and standard deviation of data were calculated from 3 measurements of 11 runs each one. The zeta potential (ζ) of NP dispersions was determined by laser Doppler electrophoresis (LDE) using a Malvern Nanosizer NanoZS Instrument. The ζ statistical average and standard deviations were calculated from 3 measurements of 20 runs each one. Finally, *scanning electron microscopy* (SEM) analysis of HNAP71 unloaded NPs was performed (**supplementary information**).

Dexamethasone encapsulation. Dexamethasone (Dx, $\geq 98\%$ pure; Aldrich, CAS Number: 50-02-2)-loaded NPs were also prepared by nanoprecipitation method. Dx (15% w/w with respect to the polymer) and the corresponding polymer (10 mgmL^{-1}) were dissolved in a mixture 8:2 (v:v) acetone:ethanol and added dropwise to the previously mentioned aqueous buffer solution at pH 4 under magnetic stirring. The final polymer concentration was 3.0 mgmL^{-1} . The solution was dialyzed against 0.1 M Acetic Acid, 100 mM NaCl aqueous solution at pH 4 for 72h in order to eliminate the organic solvent and soluble non-encapsulated Dx. The resultant NPs were filtered through $1 \mu\text{m}$ Nylon filters to remove remaining insoluble Dx.

Encapsulation efficiency (%EE) and Loading Capacity (%LC). Dx-loaded NPs were freeze dried in order to eliminate aqueous phase. The obtained white amorphous powder was dissolved in 2 ml of a mixture 8:2 (v:v) acetone:ethanol to disassembly the nanoparticulated

structure and release the encapsulated drug; and the organic solvent is let to evaporate overnight. After that, the copolymer-Dx mixture was dissolved in a mixture acetonitrile:water (v:v, 8:2) to precipitate the polymer. Samples were centrifuged at 10.000 rpm for 5 minutes at RT and supernatant was analyzed by HPLC (Dx, $\lambda_{abs} = 239$ nm). The encapsulation efficiency (*EE*) was defined as the ratio of detected experimentally and original concentration of Dx encapsulated in the inner core of the NP (**Equation 1**) and the loading capacity as the ratio of mass of Dx detected and mass of NPs (**Equation 2**).

$$\text{Encapsulation Efficiency (\%)} = \frac{[\text{Dx}]_{\text{measured}}}{[\text{Dx}]_{\text{initial}}} \times 100 \quad (1)$$

$$\text{Loading capacity (\%)} = \frac{\text{mass of Dx measured}}{\text{mass of NPs}} \times 100 \quad (2)$$

Cell culture. The RAW264.7 (murine macrophages) cell line was purchased from Sigma-Aldrich. Cells were maintained over permissive conditions in high-glucose Dulbecco's modified Eagle's medium (DMEM; Sigma, Saint Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, BRL), 2% L-Glutamine (Sigma, Saint Louis, MO, USA) and Penicillin-G (Sigma, Saint Louis, MO, USA) at 37 °C in a humidified incubator with 5% CO₂.

Uptake of c6-labelled NPs by macrophages. C6-loaded NPs were used as a model to study macrophage uptake of the NPs in cell culture. RAW264.7 cells were seeded into 6 well-plates (1.7×10^5 cells mL⁻¹), in complete DMEM. The cells were incubated overnight at 37 °C and 5% CO₂. The medium was replaced with the corresponding NP suspension in DMEM (NPs:DMEM (1:5)) and incubated over different times from 1h to 24h at 37 °C. Cells were harvested (and counted to normalize fluorescence/cell) at 1, 2, 4, 6, 8 and 24h after NPs addition, and were washed with cold PBS and centrifuged at 10.000 rpm. Supernatant was discarded and cell's pellet was lysate with ethanol to free internalized c6. Fluorescence of supernatant at 458/540 nm (excitation/emission) was measured by a Multi-Detection Microplate Reader Synergy HT (BioTek Instruments; Vermont, USA).

In vitro cytotoxicity assay of NPs. 2×10^5 live cells mL⁻¹ (100 μ L per well) were seeded in a 96 well-plate under permissive conditions. After 24h, cells were exposed to different concentrations of unloaded NAP NPs, 8Dx-NAP NPs suspension (0.25, 0.125, 0.09, 0.045, 0.023 and, 0.011 mg mL⁻¹), or free Dx; and cell viability was determined after 24h of incubation. AlamarBlue (Invitrogen) was used to determine cell viability. Absorbance at 570 nm was measured by a Multi-Detection Microplate Reader Synergy HT (BioTek Instruments; Vermont, USA). The treatments were done in replicates (n = 8). Results of the experiments were expressed as percentage of relative cell viability (% respect to the control).

Test anti-inflammatory activity of NPs

Nitric Oxide (NO) assay. Briefly, 2×10^5 live cells mL^{-1} (100 μL per well) were seeded in a 96 well-plate under permissive conditions. After 24h cells were treated with lipopolysaccharide (LPS, $5 \mu\text{g mL}^{-1}$; from Escherichia coli O111:B4; CAS Number: 297-473-0, Aldrich) and with different $[\text{NPs}]_F$ (0.125, 0.09, 0.045, 0.023, 0.011 mg mL^{-1}) of unloaded NAP NPs, 8Dx-NAP NPs or free Dx at the concentration that is encapsulated on each sample. 24h after free Dx or NPs addition, LPS-induced NO release was evaluated using Griess reagent kit (Aldrich) in order to determine the anti-inflammatory capacity of the developed formulations. Basically, during the Griess reaction dinitrogen trioxide (N_2O_3) generated from the acid-catalyzed formation of nitrous acid from nitrite (or autoxidation of NO) reacts with sulfanilamide to produce a diazonium ion which is then coupled to N-(1-naphthyl) ethylenediamine to form a chromophoric azo product that absorbs strongly at 540 nm. The treatments were done in replicates ($n = 8$). Results were expressed as percentage of relative NO released (% respect to the control, cell activated with LPS (LPS+)) normalized by the number of cells obtained by Alamar Blue assay and considering NO level of cells activated with LPS (LPS+) 100%.

RNA extraction, reverse transcription, real-time quantitative PCR (RT-qPCR). The transcription of M_1 (*Il12b*, *Il23a*, and *Tnfa*), M_2 (*Tgfb1*, and *Il10*) and pro-angiogenic (*Vegfa*) related genes were investigated by quantitative RT-PCR. RAW264.7 cells were incubated 24h with or without LPS (500 ng mL^{-1}) and either non-treated (LPS-) or treated with unloaded NAP-NPs (0.045 mg mL^{-1}), 8%Dx-NAP-NPs ($2.55 \mu\text{M}$ Dx and 0.045 mg mL^{-1} NPs) or free Dx ($2.55 \mu\text{M}$). After 24h, macrophages were washed with medium to eliminate non-internalized NPs or free Dx from the culture media and incubated in culture medium up to seven days. At day 1 and day 7, total RNA was extracted from cells using the PureLink RNA Mini Kit (Applied Biosystems) following manufacturer's instructions.^[58] RNA concentration was determined by absorbance measurements at 260 and 280 nm in a NanoDrop 1000 spectrophotometer (Thermo Scientific) and 40 ng RNA/sample were converted into cDNA using a MultiScribe reverse transcription-based reaction kit (Applied Biosystems) in the presence of a RNase inhibitor (N8080119, Applied Biosystems) in a MyCycler thermocycler (Bio-Rad; with the following temperature profile: 25 °C-10 min, 37 °C-2h, 85 °C-5 min, 4 °C ∞). Specific primers were used for quantitative PCR. The list of specific primers used (all from Sigma) is provided in **Table 5**. The reaction was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems), in an ABI PRISM 7900HT Real-Time PCR System (Applied Biosystems; with the following temperature profile: 95 °C-15 s, 60 °C-60 s, 40 cycles). Melting curves were generated in order to verify the specificity of the

amplification (15 s, from 60 °C to 95 °C). RT-qPCR expression data were analyzed according to the $2^{-\Delta\Delta C_t}$ method [58] or as $2^{-\Delta C_t}$ normalized to β -actin. For global analysis, x fold change values were translated to a heat map representation with MeV software (Institute for Genomic Research, Rockville MD). Two-way analysis of variance (ANOVA) and Tukey test were applied to calculate the differences between the values. Values of $p < 0.05$ were considered statistically significant. The levels of significance are presented as * ($p < 0.05$), ** ($p < 0.01$) and *** ($p < 0.001$). All quantifications were performed in triplicate.

Table 5. RT-qPCR primer list

Gene Symbol	Official Name	Full	Species	Forward (5'-3')	Reverse (5'-3')
<i>Il23a</i>	Interleukin-23 subunit alpha		mouse	AATAATGCTATGGCTGTTGC	CTTAGTAGATTCATATGTCCCG
<i>Tnfa</i>	Tumor necrosis factor		mouse	CTATGTCTCAGCCTCTTCTC	CATTGGGAACCTTCTCATCC
<i>Il12b</i>	Interleukin-12 subunit beta		mouse	CATCAGGGACATCATCAAAC	CTCTGTCTCCTTCATCTTTTC
<i>Vegfa</i>	Vascular endothelial growth factor A		mouse	TAGAGTACATCTTCAAGCCG	TCTTTCTTTGGTCTGCATTC
<i>Tgfb1</i>	Transforming growth factor beta-1 proprotein		mouse	GGATACCAACTATTGCTTCAG	TGTCCAGGCTCCAAATATAG
<i>Il10</i>	Interleukin-10		mouse	CAGGACTTTAAGGGTTACTTG	ATTTTCACAGGGGAGAAATC
<i>Actb</i>	Actin, beta		mouse	GATGTATGAAGGCTTTGGTC	TGTGCACITTTATTGGTCTC

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author. Detailed information of materials and methods; and results and discussion. **Table S1.** Summary of encapsulation efficiency, loading capacity and hydrodynamic properties of unloaded, coumarin-6-loaded and dexamethasone-loaded nanoparticles prepared with L-HNAP71 copolymer. **Figure S1.** Surface diagrams representing the variation of the instantaneous molar fraction of HNAP in the copolymer. **Table S2.** Quantitative real-time PCR data. Gene transcript levels of M1 and M2 markers (with or without LPS), treated with free dexamethasone (free Dx, 2.55 μM), Dx-loaded naproxen-bearing NPs (8Dx-NAP NPs, 2.55 μM Dx and 0.045 mg mL^{-1} NPs) and unloaded naproxen-bearing NPs (NAP NPs, 0.045 mg mL^{-1}) for 1 day or 7 days. Data are presented as mean and standard deviation (mean $\pm$ SD) of the average log2 variation compared to untreated cells (LPS-) in 3 independent experiments, each quantified in triplicate (see **Figure 8 and 9**).

Conflict of interest: Authors claim that there exist no conflicts of interest.

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Table of Content (TOC)

This paper describes the preparation of new self-assembled bioactive naproxen (NAP)-containing polymeric NPs loaded with dexamethasone (Dx). Their potential to achieve spatiotemporal co-localization of Dx/NAP combination at inflamed areas through fast uptake by macrophages was demonstrated as well as, the synergistic reduction of *Il12b* transcript levels and, hence, the reduction of IL12 and IL23 levels, key players in autoimmune diseases.

IL-12-p40 subunit

Eva Espinosa-Cano, Maria Rosa Aguilar*, Yadileiny Portilla, Domingo F. Barber, Julio San Román

Polymeric nanoparticles that combine dexamethasone and naproxen for the synergistic inhibition of *Il12b* transcription in macrophages

Supporting Information

Polymeric nanoparticles that combine dexamethasone and naproxen for the synergistic inhibition of IL12b in macrophages

Eva Espinosa-Cano, Maria Rosa Aguilar, Yadileiny Portilla, Domingo F. Barber, Julio San Román*

Materials and methods

Synthesis and characterization of copolymers of naproxen-based methacrylic monomer and 1-vinyl imidazole, poly(HNAP-co-VI).

Proton Nuclear Magnetic Resonance ($^1\text{H-NMR}$) of the obtained copolymers were performed in a Varian Mercury apparatus operating at 400 MHz. Spectra were recorded by dissolving the samples in DMSO- d_6 at 25 °C. Copolymers composition was calculated using MestreNova 9.0 from the $^1\text{H-NMR}$ integral between 7.92-6.47 ppm corresponding to the aromatic protons of both monomers and the integral between 2.28-0.1 ppm which corresponds to protons of the methyl groups i and m of HNAP and protons l and n from the main hydrophobic carbon chain of HNAP and VI, respectively (**figure 2**).

Size exclusion chromatography (SEC). Copolymers apparent average molecular weight (M_n and M_w) and polydispersity index ($\mathcal{D}$) were determined by *size exclusion chromatography (SEC)*, using a Perkin-Elmer Isocratic LC pump 250 coupled to a refraction index detector (Series 200). Two Resipore columns (250 mm x 4.6 mm, Varian) were used as solid phase, and degassed chromatographic grade dimethylformamide (DMF, Scharlab, 0.7 mLmin $^{-1}$) with LiBr (0.1 % w/v) was used as eluent and temperature was fixed at 70 °C. Monodisperse PMMA standards (Scharlab) with molecular weights between 10,300 and 1,400,000 Da were used to obtain the calibration curve. Data was analyzed using the Perkin-Elmer LC solution program.

Differential scanning calorimetry (DSC). Glass transition temperatures (T_g) were determined by *differential scanning calorimetry (DSC)* with a Perkin Elmer DSC8500 interfaced to a Pyris thermal analysis data system. Dried samples (3-5 mg) were placed in aluminium pans and heated from -20 to 120 °C (200 °C in case of poly(VI)) at a constant speed of 20 °Cmin $^{-1}$. T_g was taken as the midpoint of the heat capacity transition.

Preparation and characterization of self-assembled nanoparticles**Scanning electron microscopy NPs morphology characterization**

To determine NPs shape, a SEM analysis of HNAP71 unloaded NPs was performed with a Hitachi SU8000 TED, cold-emission FE-SEM microscope working with an accelerating voltage 1 kV-D. Sample preparation consisted in the deposition of one drop of the corresponding NPs suspension (0.02 mgmL^{-1}) over a small glass disk (12 mm diameter) and evaporation at room temperature. Gold palladium alloy (80:20) was used to coat the samples prior to examination by SEM.

Optimization of final NP concentration ($[NPs]_F$) and final volume (V_F)

Different final concentrations of NPs in aqueous suspension ($[NPs]_F = 1 \text{ mgmL}^{-1}$ and 5 mgmL^{-1}) and three different final volumes of the aqueous phase ($V_F = 10 \text{ mL}$, 20 mL and 30 mL) were tested. Hydrodynamic properties were measured to determine the largest volume and final concentration that can be prepared keeping hydrodynamic properties within the appropriate ranges for cell uptake by inflammatory cells.

Optimization of storage conditions

A *pH* study was performed to determine the aggregation *pH* of the systems and set the appropriate storage *pH*. NPs were prepared at *pH* = 4.0 and hydrodynamic properties were measured. *pH* was increased by 0.5 via the addition of 1 M NaOH aqueous solution and size distribution and ζ were measured. The procedure was repeated until the aggregation *pH* was reached. After that, the *pH* was set to 4.0 again to check reversibility of the aggregation phenomenon. Samples size distribution and ζ were measured every week up to one month to demonstrate NPs stability when stored in suspension at 4°C and at the most suitable storage *pH*. NPs were freeze-dried after preparation and dispersed in the same volume of 0.1 M acetic acid buffer solution. The suspension was then subjected to manual shaking and ultrasound bath/tip sonication (30 % amplitude) for 10 minutes. After sonication, size distribution of NPs was measured to check redispersibility.

Preparation of c6-loaded NPs

Coumarin-6(c6)-loaded NPs were also prepared by nanoprecipitation method. c6 (Aldrich, 1% w/w with respect to the polymer) and the corresponding polymer (10 mgmL^{-1}) were dissolved in a mixture 8:2 (v:v, acetone:ethanol) and added dropwise to the aqueous buffer solution (0.1 M NaCl, 0.1 M Acetic Acid) at *pH* 4 under magnetic stirring. The final polymer concentration was 3.0 mgmL^{-1} . The solution was dialyzed against the same aqueous buffer

solution at *pH* 4 for 72h in order to eliminate the organic solvent and soluble non-encapsulated c6. The resultant NPs were filtered through 1 μ m Nylon filters to remove remaining insoluble c6.

C6 encapsulation efficiency (%EE) and Loading Capacity (%LC)

c6-loaded NPs were freeze dried in order to eliminate aqueous phase. The obtained white amorphous powder was dissolved in 2 ml of a mixture 8:2 (v:v) acetone:ethanol to disassembly the nanoparticulated structure and release the encapsulated drug; and the organic solvent is let to evaporate overnight. After that, the copolymer-c6 samples in ethanol to precipitate the polymer. Samples were centrifuged at 10.000 rpm for 5 minutes at RT and supernatant was analyzed by UV spectrophotometry (c6, λ_{abs} = 459 nm). The encapsulation efficiency (*EE*) was defined as the ratio of detected experimentally and original concentration of c6 encapsulated in the inner core of the NP (**Equation 1S**) and the loading capacity as the ratio of mass of c6 detected and mass of NPs (**Equation 2S**).

$$\text{Encapsulation Efficiency (\%)} = \frac{[c6]_{\text{measured}}}{[c6]_{\text{initial}}} \times 100 \quad (1S)$$

$$\text{Loading capacity (\%)} = \frac{\text{mass of c6 measured}}{\text{mass of NPs}} \times 100 \quad (2S)$$

Results

C6-loaded NPs to monitor cellular uptake of NPs

C6 was used as a fluorescent probe in NPs internalization cellular studies. HNAP71-based NPs encapsulate c6 with low loading capacity (%LC = 0.92) to avoid fluorescence quenching of the signal. Encapsulation efficiency higher than 90% was achieved (Encapsulation Efficiency (%EE) = 92%) and only an increase in 10 nm on the NPs diameter was observed with no significant changes in surface charge (**table S1**).

Table S1. Summary of encapsulation efficiency, loading capacity and hydrodynamic properties of unloaded, coumarin-6-loaded and dexamethasone-loaded nanoparticles prepared with L-HNAP71 copolymer

Acronym	Sample	EE ^{a)} [%]	LC ^{b)} [%]	D_h ^{c)} [nm]	<i>Pdl</i> ^{d)}	ζ ^{e)} [mV]
NAP NPs	NP-L-HNAP71	-	-	131.5 $\pm$ 3.3	0.115 $\pm$ 0.016	+ 28.8 $\pm$ 0.7
Dx-NAP NPs	8Dx-NP-L-HNAP71	8	2.00	149.5 $\pm$ 1.8	0.085 $\pm$ 0.018	+ 26.1 $\pm$ 0.9
c6-NAP NPs	92c6-NP-L-HNAP71	92	0.92	140.0 $\pm$ 1.1	0.132 $\pm$ 0.026	+ 28.9 $\pm$ 0.6

^{a)}Encapsulation Efficiency, ^{b)}Loading capacity, ^{c)}hydrodynamic diameter, ^{d)}polydispersity and ^{e)}zeta potential

Figure S1. Surface diagrams representing the variation of the instantaneous molar fraction of HNAP in the copolymer.

Table S2. Quantitative real-time PCR data. Gene transcript levels of M_1 and M_2 markers (with or without LPS), treated with free dexamethasone (free Dx, $2.55 \mu\text{M}$), Dx-loaded naproxen-bearing NPs (8Dx-NAP NPs, $2.55 \mu\text{M}$ Dx and 0.045 mg mL^{-1} NPs) and unloaded naproxen-bearing NPs (NAP NPs, 0.045 mg mL^{-1}) for 1 day or 7 days. Data are presented as mean and standard deviation (mean $\pm$ SD) of the average log2 variation compared to untreated cells in 3 independent experiments, each quantified in triplicate (see **Figure 8a**, **figure8b** and **figure 9a**).

M ₁ and M ₂ markers (LPS-)						
Genes	Free Dx		8Dx-NAP NPs		NAP NPs	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
<i>Il23a</i> (M_1)	0.40 ± 0.21	0.82 ± 0.45	0.07 ± 0.02	0.29 ± 0.13	-0.18 ± 0.10	0.37 ± 0.24
<i>Il12b</i> (M_1)	-0.19 ± 0.06	-0.18 ± 0.08	-12.28 ± 0.04	-12.19 ± 0.27	-0.34 ± 0.17	0.05 ± 0.01
<i>Tnfa</i> (M_1)	0.16 ± 0.07	0.51 ± 0.01	0.04 ± 0.01	0.67 ± 0.40	-0.09 ± 0.01	0.46 ± 0.33
<i>Vegfa</i> (M_2)	0.28 ± 0.16	-1.22 ± 0.03	0.42 ± 0.35	-0.04 ± 0.02	-0.28 ± 0.17	0.09 ± 0.01
<i>Tgfb1</i> (M_2)	0.13 ± 0.03	-0.15 ± 0.07	-0.08 ± 0.02	-0.02 ± 0.01	-0.56 ± 0.50	0.04 ± 0.02
<i>Il10</i> (M_2)	-1.70 ± 0.84	-0.89 ± 0.03	-0.15 ± 0.05	-0.43 ± 0.20	-0.40 ± 0.21	0.05 ± 0.01

M ₁ markers (LPS+)								
Genes	CP (+LPS)		Free Dx		8Dx-NAP NPs		NAP NPs	
	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7	Day 1	Day 7
<i>Il23a</i>	1.54 ± 0.03	-2.78 ± 0.25	0.69 ± 0.12	0.24 ± 0.06	0.53 ± 0.38	0.88 ± 0.10	1.54 ± 0.33	-2.77 ± 0.07

<i>Il12b</i>	0.17 ± 0.02	3.51 ± 0.01	-0.40 ± 0.01	-0.06 ± 0.01	-12.33 ± 0.01	-12.1 ± 0.28	0.17 ± 0.02	3.51 ± 0.14
<i>Tnfa</i>	3.70 ± 0.28	0.91 ± 0.01	0.60 ± 0.12	0.59 ± 0.21	0.73 ± 0.12	1.18 ± 0.10	3.71 ± 0.70	0.91 ± 0.03