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Nanoparticle-encapsulated $P2X_7$ receptor antagonist in a pH-sensitive polymer as a potential local drug delivery system to acidic inflammatory environments

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ABSTRACT

We have developed nanoparticles of anti-inflammatory $P2X_7$ receptor antagonist encapsulated in a pH-sensitive polymer, poly(tetrahydropyran-2-yl methacrylate) (poly(THPMA)), as a potential local drug delivery system to target to acidic inflammatory environments, in which $P2X_7$ receptors are implicated in the pathology of inflammation via the activation of immune cells. The nanoparticles were prepared using single emulsion methods, also their size and shape were confirmed by microscopy and spectroscopy, etc. The profiles of the pH-dependent degradation, release of antagonist and biological activities were investigated. The nanoparticles that encapsulated the 3,5-dichloropyridine derivative (**2**) with poly (THPMA), were observed to be more slowly cleaved than the blank nanoparticles. Moreover, the free $P2X_7$ receptor antagonists potently inhibited the receptor activation, whereas the nanoparticles of the 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) exhibited much lower $P2X_7$ antagonistic activity through sustained encapsulation. Thus, the nanoparticles of the 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) may be utilized to develop a pH-sensitive local drug delivery system for controlled release of anti-inflammatory therapeutics in acidic physiological environments.

The purinergic $P2X_7$ receptor is a ligand-gated ion channel, that is, activated by ATP binding. The $P2X_7$ receptor is widely expressed in cells of the central and peripheral nervous systems, including microglia, macrophages, and the retina.[1] The $P2X_7$ receptor has been reported to be involved in inflammation and chronic pain through the activation of immune cells, suggesting that the receptor is a promising therapeutic target of these diseases.[2] The target validation of the $P2X_7$ receptor has been established by studies on $P2X_7$-knockout animals, which exhibited resistance to inflammation and neuropathic pain.[3] For spinal cord injuries, the potential utilization of $P2X_7$ receptor antagonists as a novel therapeutic approach was confirmed by a report describing the increased level of $P2X_7$ receptor expression and ATP concentration in the region of injury and the striking recovery of the injury following treatment with a $P2X_7$ receptor antagonist, oxidized ATP.[2] Additionally, significant attention has been given to the close relationship between the $P2X_7$ receptor and joint inflammation, as occurs in rheumatoid arthritis, because the activation of the $P2X_7$ receptor mediates IL-1β release, which is an important cytokine in joint inflammatory diseases.[4] Therefore, the etiological factors of rheumatoid arthritis for inflammation affect the targeting factors involving $P2X_7$ receptors.

Hence, $P2X_7$ receptor antagonists have been extensively developed for the treatment of inflammation, neurodegeneration and chronic pain based on the pathological functions of the receptor upon activation by a high concentration of ATP in acidic inflammatory environments.[5] KN-62 (**1**) is one of the early $P2X_7$ receptor antagonists and is a potent *h*$P2X_7$ receptor antagonist, with an IC_{50} value of 51 nM.[6] Despite the potent antagonistic effect of KN-62 (**1**) on the $P2X_7$ receptor, this antagonist exhibits several disadvantages such as poor water solubility[7] and noncompetitive antagonistic activity[8] that limit its development as a drug. Our group has developed a 3,5-dichloropyridine derivative (**2**) as a novel $P2X_7$ receptor antagonist with an IC_{50} value of 13 nM for the *h*$P2X_7$ receptor in ethidium bromide (EtBr) uptake assays. Moreover, this novel $P2X_7$ receptor antagonist exhibits potent inhibitory activity in the release of the proinflammatory cytokine IL-1β, iNOS/COX-2 expression and NO production in LPS/IFN-γ/BzATP-stimulated THP-1 cells, suggesting that the 3,5-dichloropyridine

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derivative (**2**) may be a suitable anti-inflammatory agent targeting the $P2X_7$ receptor.[9]

Among the $P2X_7$-receptor-related diseases, rheumatoid arthritis is a systemic inflammatory disorder that affects several tissues and organs but principally attacks synovial joints. Although rheumatoid arthritis is an autoimmune disease, one of the major players in this disease is IL-1β, which is released following activation of the $P2X_7$ receptor.[10] The region of joint inflammation is known to be an acidic environment because rheumatoid arthritis evokes the activation of a metabolic byproduct,[11] which results in an imbalance between the increased metabolic activity and insufficient vascular supply, leading to the induction of anaerobic glycolysis to form lactate and H^+.[12] Hence, the pH of inflammatory arthritic tissue is observed to be approximately 5.1 compared with a pH of 7.4 for normal tissues in a physiological environment.[13]

Due to this unique pathophysiological feature of inflammation, as occurs in rheumatoid arthritis, our study aimed at targeted therapy using encapsulated agents, that is, novel $P2X_7$ receptor antagonists that have been previously identified by our group as anti-inflammatory drug candidates, in pH-sensitive polymers for selective degradation in the acidic environment of the inflammatory region.[14] The pH-sensitive biodegradable polymers, poly(tetrahydropyran-2-yl methacrylate) (poly(THPMA)),[15] poly[2-(*N*,*N*-diethylamino)ethyl methacrylate] (PDMAEMA)[16] and poly(β-amino ester) derivatives,[17] have been developed in several studies. Among these polymers, we selected poly(THPMA) as the pH-sensitive polymer because the major degradation product under acidic conditions, poly(methacrylic acid) (PMAA), is nontoxic and water soluble.[15]

In this study, we report nanoparticle-encapsulated $P2X_7$ receptor antagonist in a pH-sensitive polymer as a potential local drug delivery system. We describe the preparation of the nanoparticles, which encapsulate anti-inflammatory drug candidate, as well as the dissolution and release of the drugs under acidic conditions (pH 5.1 to 6). We prepared nanoparticles using 3,5-dichloropyridine derivative (**2**) as a $P2X_7$ receptor antagonists. For clarification of the development of this passive targeting drug delivery system for an acidic inflammatory environment, we compared the weight change of the nanoparticles and the in vitro release of encapsulated agent at pH 5.1 and pH 7.4, respectively. The biological activity was also examined. A SRB assay was performed to assess the cytotoxicity in *h*$P2X_7$-expressing HEK-293 cell line and validate the antagonistic effect on the $P2X_7$ receptor using an EtBr uptake assay and *h*$P2X_7$-expressing HEK-293 cell. These nanoparticles represent a potential local drug delivery system to acidic inflammatory environments with more selective and effective release at lower pH. Based on these pathological features, we propose a novel type of nanoparticle for passive targeting to minimize the side effects of anti-inflammatory drugs and to overcome the inherent properties of the drug, such as pharmacokinetic problems (Fig. 1).

We synthesized 3,5-dichloropyridine derivative (**2**) as following below scheme (Fig. 2). 3,5-Dichlroro-4-iodopyridine and hydrazine hydrate were refluxed and then amide coupling reaction was occurred.[9] Poly(tetrahydropyran-2-yl methacrylate) (poly (THPMA)), a pH-sensitive polymer, was polymerized by radical reaction using AIBN from tetrahydro-2H-pyran-2-yl methacrylate, monomer of poly(THPMA), which is synthesized from esterification of methacrylic acid with 3,4-dihydro-2*H*-pyran by *p*-toluenesulfonic acid monohydrate.[15] After synthesis of antagonist and polymer, we confirmed structure by ^{1}H NMR, mass spectrometry and molecular weight was measured by GPC, these data were shown in notes.[18] Encapsulated nanoparticle and blank nanoparticle were prepared by single emulsion method. Single emulsion method is given sonicated power only once at oil/water condition for fabricating micelle. First of all, 3,5-dichloropyridine derivative

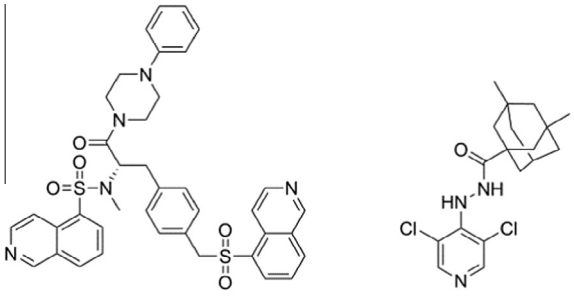
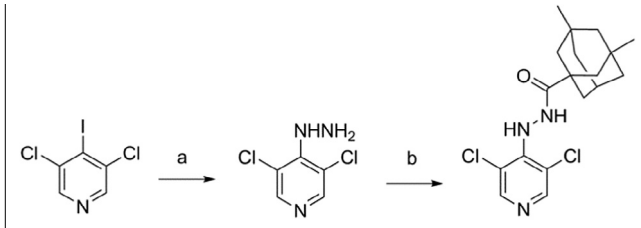
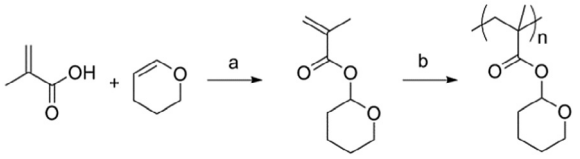


Figure 1. Antagonists of the $P2X_7$ receptor. KN-62 (**1**) is a potent *h*$P2X_7$ receptor antagonist, with an IC_{50} value of 51 nM.[6] The 3,5-dichloropyridine derivative (**2**) is a novel $P2X_7$ receptor antagonist, with an IC_{50} value of 13 nM for *h*$P2X_7$ receptors in an ethidium bromide uptake assay developed by our group.[9]



Reagents and conditions: (a) hydrazine hydrate, EtOH, 90°C, 16 h, 78%; (b) EDC, DIPEA, 3,5-dimethyladamatylcarboxylic acid, DCM, rt, 4 h, 93%



Reagents and conditions: (a) *p*-TsOH, DCM, rt, 20 min (b) AIBN, THF, 70°C, 12 h

Figure 2. Scheme of 3,5-dichloropyridine derivative (**2**) and poly(THPMA).

(**2**) and poly(THPMA) were dissolved in dichloromethane. The mixture was added to aqueous solution of polyvinyl alcohol (PVA) and subsequently emulsified using a probe sonicator at 80 W for 2 min to form primary emulsion that was soon added to the second aqueous PVA solution, followed by stirring at room temperature for 4 h until evaporation of the organic solvent was complete. The resulting nanoparticles were collected by centrifugation at 13,000 rpm for 20 min at 4 °C, re-suspended in cold water, and centrifuged again to remove excess PVA. Finally, the nanoparticles were lyophilized to yield a white, fluffy powder. Moreover, the blank nanoparticles were prepared by single emulsion method same as poly (THPMA) nanoparticles.

To confirm of characterization of $P2X_7$ receptor antagonist-encapsulated nanoparticles, first, we determined particle size which is directly mean diameter by photon correlation spectroscopy (PCS) using water solvent dispersion, and also, surface and morphology analyzed by means of scanning electron microscopy (SEM) examination. To quantify the amount of drug loaded in nanoparticles, calibration curves for each drug were determined using UV spectroscopy at the 250 nm wavelength (the detailed procedure was described in notes and the calibration curves are provided in the Supporting materials).[19] We measured the weight loss of the blank or poly(THPMA) nanoparticles encapsulated 3,5-dichloropyridine derivative (**2**) at pH values corresponding to

acidic inflammatory tissue (pH 5.1, 100 mM acetate buffer) and the normal physiological condition (pH 7.4, 100 mM HEPES buffer) to check of pH-dependent degradation of the nanoparticles. The nanoparticles were dispersed in each buffer at a concentration of 2 mg/mL. The suspensions were incubated at 37 °C with mixing for 4 days. At 0, 24, 48 and 96 h, 5 mL of the suspension were collected. The nanoparticles were re-suspended in water and lyophilized. The lyophilized nanoparticles were collected and weighed. At a regular interval, we measured the amount of released $P2X_7$ antagonist following degradation of the poly(THPMA) nanoparticles encapsulating 3,5-dichloropyridine derivative (**2**) at pH 5.1, as an acidic inflammatory condition, and at pH 7.4 for 8 days. These release experiment was performed for elucidating the amount of the drug accumulation via in vitro system as a function of different pH.

The poly(THPMA) molecular weight and the poly-dispersity regarding the type of polymer structure were determined to be 28,276 and 1.371, respectively. The synthesized polymer was determined to be a more uniform structure based on the poly-dispersity value near 1. The overall size of each nanoparticle ranged from 300 to 400 nm, which lies within the nanoscale range (Table 1). The size of the poly(THPMA) nanoparticles without drug encapsulation was 340 nm. The poly(THPMA) nanoparticles encapsulating the 3,5-dichloropyridine derivative (**2**), which used for the single emulsion method, were slightly larger (363 nm) than the blank nanoparticles. The percentages of the loaded drug are summarized in Table 1. In general, 3,5-dichloropyridine derivative (**2**) was encapsulated more than 64.6% in the poly(THPMA) nanoparticles. We confirmed that each nanoparticle possessed a globular shape with a smooth surface without distorted holes, as shown in Figure 3.

Table 1
Characterization of nanoparticles

Nanoparticles	Encapsulation efficiency (%)	Particle size[b] (nm)
Blank poly(THPMA)	—[a]	340 ± 82 [c]
Poly(THPMA) encapsulated 3,5-dichloropyridine derivative (**2**)	64.6	363 ± 67 [d]

[a] Any agent was not loaded in blank poly(THPMA) nanoparticles.
[b] Each nanoparticles were determined particle size by photon correlation spectroscopy (PCS) at three times.
[c] Blank poly(THPMA) nanoparticles had 1.3311 of refractive index and the scattering intensity was 11559 (cps).
[d] Poly(THPMA) nanoparticles encapsulated 3,5-dichloropyridine derivative (**2**) had 1.3312 of refractive index and the scattering intensity was 11734 (cps).

Using the blank poly(THPMA) and 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) nanoparticles, the rate of degradation and morphological change of the nanoparticles were studied at pH 5.1 and 7.4, which correlate with an acidic inflammatory and normal physiological environment, respectively. In Figure 4a, morphological changes of 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) nano-particles for 0 to 2 days at different pH values (pH 7.4 and pH 5.1). At the point of 1 day, each different pH manner had almost same degradation rate, but time goes by, the rate of degradation was faster in pH 5.1 than normal condition pH 7.4. Acidic environments cause the nanoparticles to degrade and release the encapsulated drug more rapidly than under normal pH conditions for similar processing times. After 2 days of incubation, the rate of degradation of the blank poly(THPMA) (80%) was observed to be more rapid than that of the 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) (50%) at pH 5.1 (Fig. 4b and c). The degradation rates of 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) at pH 5.1 and pH 7.4 after 4 days were observed 70% and 40%, respectively. These results indicate that the nanoparticles encapsulating the agent were more slowly degraded, and the release time could be controlled using the passive targeting drug delivery system.

At a regular interval, we measured the amount of released $P2X_7$ antagonists by HPLC analysis following degradation of the 3,5-dichloropyridine derivative (**2**) encapsulated poly (THPMA) nanoparticles at pH 5.1, as an acidic inflammatory condition, and at pH 7.4 for 8 days.[20] Although nearly 70% of the nanoparticles were degraded after 4 days at pH 5.1, as illustrated in Figure 4b and c, which show the weight change of whole particles from 0 to 4 days, only 20% of the 3,5-dichloropyridine derivative (**2**) was released during this period (Fig. 5). The release of compound (**2**) was completed in 8 days at pH 5.1, whereas only 35% of compound (**2**) was released at pH 7.4 even after 8 days.

In a cell proliferation assay to evaluate the cytotoxicity, the poly (THPMA) nanoparticles exhibited of no cytotoxic effect in the *h*$P2X_7$-expressing HEK-293 cell line up to 10 μM. The detailed data is shown in Supporting materials.[21] Next, the $P2X_7$ receptor antagonistic activity of the nanoparticles was determined. As shown in Figure 6 and 100 nM of each free agent and the poly(THPMA) nanoparticles, 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) and KN-62 (**1**) as a positive control, were evaluated.[22] The free forms of KN-62 (**1**), 3,5-dichloropyridine derivative (**2**) exhibited potent $P2X_7$ receptor antagonistic activity, with 45% and 90% inhibition effect, respectively, at a concentration of 100 nM,

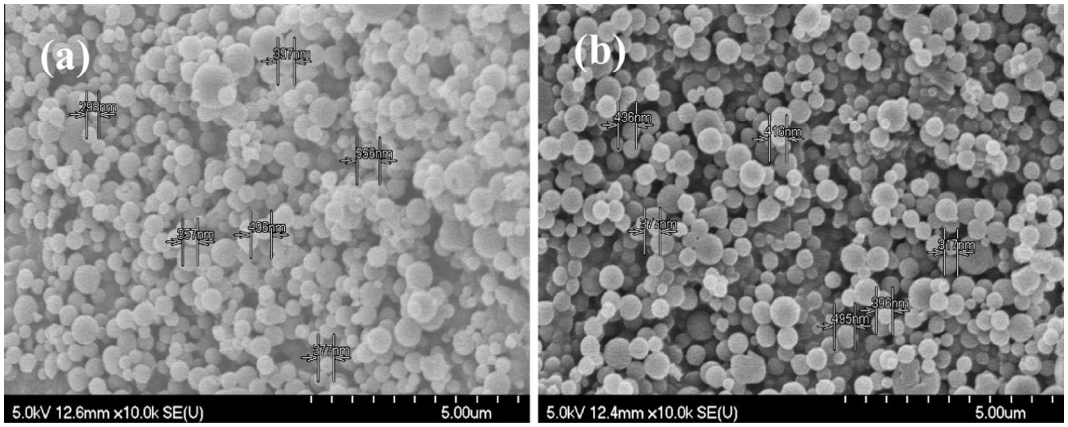


Figure 3. Measurement of the particle size, surface and morphology analysis for nanoparticle characterization. (a) Blank poly(THPMA) nanoparticles, (b) poly(THPMA) nanoparticles which encapsulated 3,5-dichloropyridine derivative (**2**).

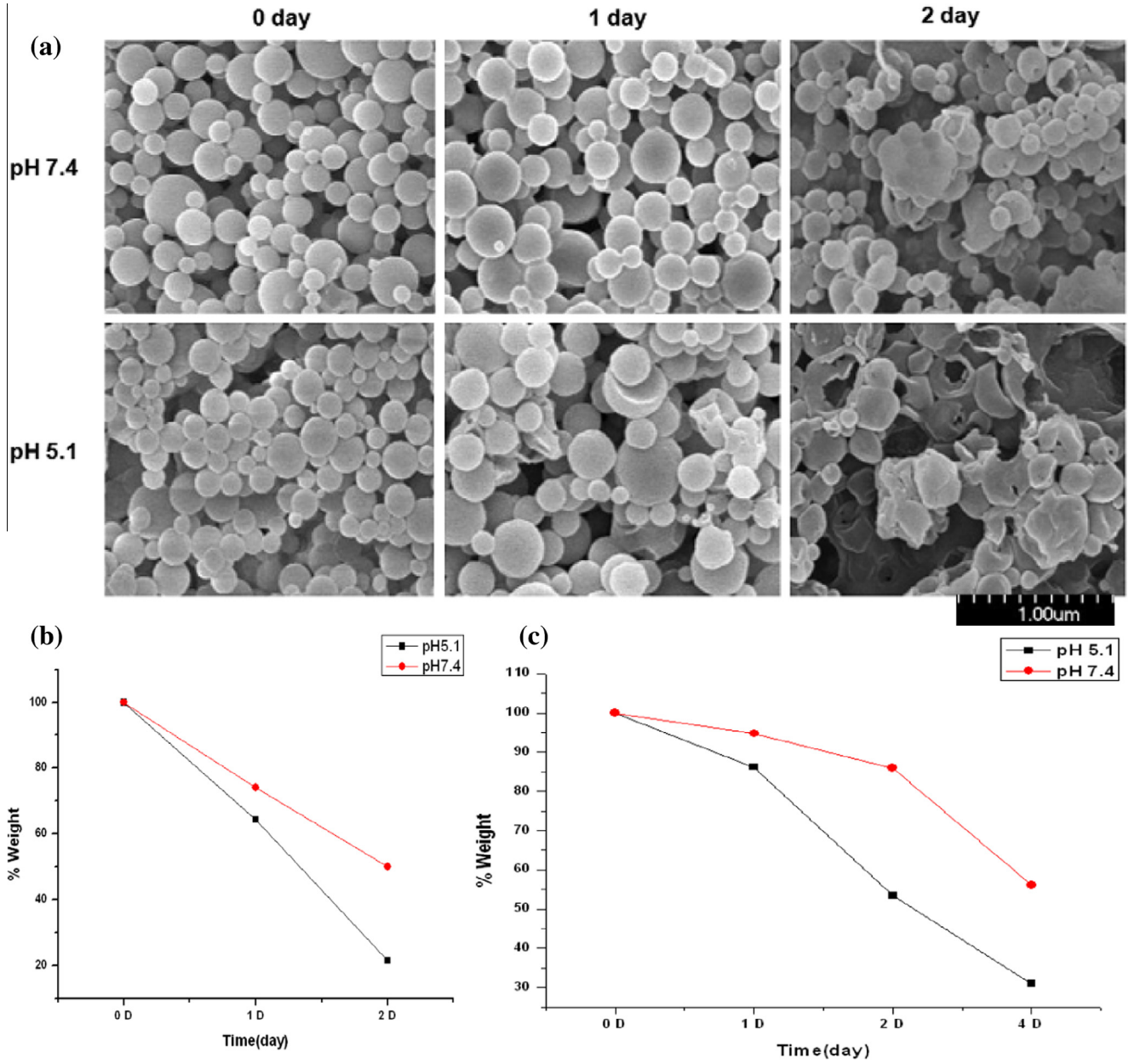


Figure 4. pH-Dependent degradation of the nanoparticles. (a) Morphological changes of 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) nanoparticles for 0 to 2 days at different pH values (pH 7.4 and pH 5.1). (b and c) Total weight change of blank poly(THPMA) and 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) nanoparticles, respectively, at pH 5.1 and pH 7.4.

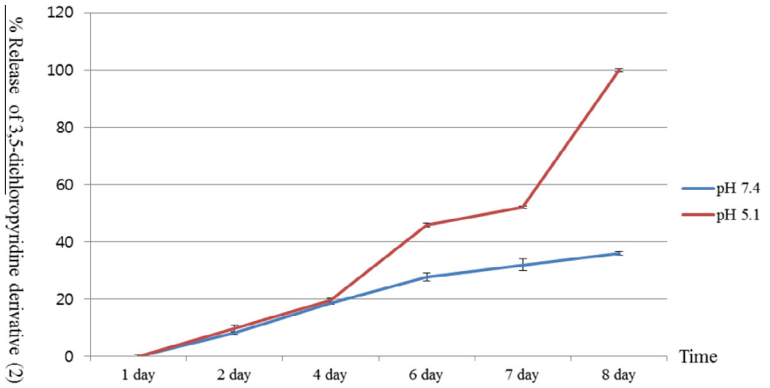


Figure 5. Percent release of 3,5-dichloropyridine derivative (**2**) from nanoparticles in a pH-dependent manner as a function of processing time. Every test was extracted more than 3 times and statistics were shown in error bar.

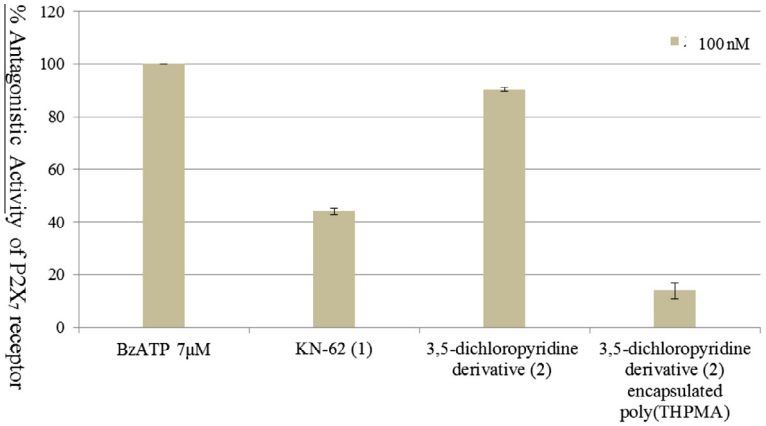


Figure 6. $P2X_7$ receptor antagonistic effect of the nanoparticles in an ethidium bromide uptake assay. Every test was examined each 3 times and statistics were shown in error bar.

which are consistent with previously reported values.[6,9] The 3,5-dichloropyridine derivative (**2**) encapsulated poly(THPMA) nanoparticles exhibited significantly low antagonistic activity, with only 15% inhibition at a concentration that corresponded to 100 nM of the free agent.

An important issue in determining the efficiency of a drug delivery system is the ability to control the exact location and specific time at which the drugs are released. This challenge has motivated the development of particulate nanometer systems using pH-sensitive biodegradable polymers, poly(THPMA), that are designed to release loaded anti-inflammatory agents in a pH-dependent manner at the target site of inflammation, which is generally an acidic environment. The nanoparticles formed by poly(THPMA) are attractive for use as a potential local delivery system for $P2X_7$ receptor antagonists that demonstrate potential anti-inflammatory activity under identified pathological conditions in an acidic inflammatory environment through the triggered facilitative release in response to different pH environmental stimuli. In this study, we confirmed that the blank poly(THPMA) nanoparticles degraded more rapidly at both pH 5.1 and pH 7.4 than the $P2X_7$ receptor antagonist-encapsulated nanoparticles, 3,5-dichloro-pyridine derivative (**2**) encapsulated poly(THPMA), suggesting that the nanoparticle-encapsulated drug could be released in an acidic inflammatory environment over a certain time period. In the evaluation of the receptor antagonistic activity, the free $P2X_7$ receptor antagonists, KN-62 (**1**) and the 3,5-dichloropyridine derivative (**2**) potently inhibited the receptor activation, whereas the nanoparticles of the 3,5-dichloropyridine derivative (**2**) encapsulated poly (THPMA) exhibited much lower $P2X_7$ antagonistic activity, suggesting that the nanoparticles could sustain encapsulation in a cell culture environment at pH 7.2–4.

In conclusion, we have developed a drug delivery system consisting of nanoparticles containing an anti-inflammatory hydrophobic $P2X_7$ receptor antagonist, 3,5-dichloropyridine derivative (**2**), encapsulated in pH-sensitive poly(THPMA) to provide more efficient local treatment of inflammatory conditions through the controlled release of anti-inflammatory therapeutics in acidic physiological environments such as inflamed tissues at local arthritic lesions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2015.08.004.

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18. *Spectroscopy data of 3,5-dichloropyridine derivative (**2**) and poly (THPMA) (NMR: $CDCl_3$, 400 MHz) δ (ppm) J (Hz)*: 8.29 (s, 2H), 7.82 (d, J = 4.0, 1H), 6.79 (d, J = 4.4, 1H), 2.14–2.13 (m, 1H), 1.71 (d, J = 2.8, 2H), 1.51 (q, J = 12.6, 4H), 1.51 (q, J = 10.6, 4H), 1.36–1.15 (m, 2H), 0.85 (s, 6H). ESI [M+H] = 369.8. Poly(THPMA)

6.17 (quint, *J* = 4, 1H) 6.04 (t, *J* = 4, 1H) 5.60 (quint, *J* = 4, 1H) 3.87 (m, 1H) 3.71 (m, 1H) 1.95 (t, *J* = 4, 3H) 1.84 (m, 2H) 1.73 (m, 2H) 1.54 (m, 2H). Mw = 28216. Poly-dispersity = 1.371.

19. *Determination of the drug loading efficiency*: The drug, 3,5-dichloropyridine derivative (**2**) was dissolved in methanol, and a calibration curve was determined at the concentrations of 10, 20, 30, 40 and 50 μg/mL. Following dissolution, the UV absorption of the solution was examined using a spectrofluorometer to estimate the amount of agent loaded in the nanoparticles. The drug encapsulation efficiency of the nanoparticles was determined by measuring the amount of free drug using a direct method. In this approach, 1 mg of each nanoparticle was dissolved in DCM, subsequently, saturated $NaHCO_3$ was added to the solution, which was stirred for 6 h at room temperature. After stirring, the DCM layer was extracted 3 times, and the DCM was evaporated to obtain the drug, which was encapsulated by the nanoparticles. The obtained drug was dissolved in methanol (1 mL), and the 250 nm wavelength of UV absorption was measured using a spectrofluorometer. Finally, the measured UV absorption was compared with the calibration curve of each drug to estimate the amount of encapsulated drug.
Encapsulation efficiency (%) = (The amount of drug encapsulated in the nanoparticles)/(The amount of drug used) × 100
20. *In vitro drug accumulation test*: A release experiment was performed in 100 mM HEPES, pH 7.4, and 100 mM acetate, pH 5.1. For this experiment, 60 mg of each type of dried nanoparticles, 3,5-dichloropyridine derivative (**2**) @ poly(THPMA) was transferred into a tube and dispersed in 30 mL of HEPES buffer and acetate buffer, respectively, followed by incubation at 37 °C with mixing at 150 rpm. At appropriate 24-h intervals, 1 mL of the suspension was collected and centrifuged for 30 min at 14,000 rpm. Subsequently, 100 μL of the supernatant was collected, and 900 μL of ethyl acetate was added for subsequent fluorescence analysis. The UV absorption of 1 mL of the resultant solution was measured using a spectrofluorometer at 250 nm wavelength.
21. *Cell cytotoxicity assay*: The cytotoxicity effect of the (**2**) encapsulated poly (THPMA) nanoparticles and compound (**2**) were determined by a sulforhodamine B colorimetric assay (SRB assay). The *h*$P2X_7$-expressing HEK-293 cell were seeded into 96-well plate at a density of 5×10^4 cells/well in 100 μL of medium and (**2**) encapsulated poly(THPMA) nanoparticles and compound (**2**) were treated by concentration of 10 and 1 μM then incubated for 3 days at 37 °C in a humidified atmosphere of 5% CO_2-95% air. After 3 days incubation, 50 μL of 50% trichloroacetic acid (TCA) was added each well and incubated for 40 min at 4 °C to fix cell. Each well was washed by distilled water 5 times then dried. A 100 μL of 0.4% sulforhodamine B reagent (in 1% of acetic acid) was added for 1 h at room temperature for staining. The wells were washed with 1% acetic acid 5 times for removing dye, then dried, and 10 mM tris(hydroxymethyl)aminomethane (tris base, pH 10) 200 μL was treated to each well. After dissolving remaining dye, the resultant solutions were measured using microplate reader at 515 nm (FL600, Bio-Tek Inc., Winooski, VT). The data are expressed as the protein of viable cells percentage compared to control group.
22. *Ethidium accumulation in hP2X7-expressing HEK-293 cell line*: All experiments were performed using adherent HEK-293 cells stably transfected with cDNA encoding the human $P2X_7$ receptor. Either or the each concentration of free drug and nanoparticles were treated to each well of 96 well. *h*$P2X_7$-expressing HEK-293 cells were then re-suspended at 2.5×10^6 cells/mL in HEPES-buffered salt solution that comprised (in mM) : ethidium bromide 0.1, ethylene diamine tetraacetic acid (EDTA) 1, glucose 5, HEPES 20, potassium chloride 140 (pH 7.4). The cell suspension was treated to the wells of 96 well plate followed by addition of BzATP of 7 μM concentration. The plates were incubated at 37 °C for 120 min and cellular accumulation of ethidium$^+$ was determin-ed by measuring fluorescence with fluorescent plate reader (excitation filter of 530 nm and emission filter of 590 nm).