Journal of Research in Pharmacy

Research Article

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Development and *in-vitro* evaluation of chitosan chloride decorated PLGA based polymeric nanoparticles of nimesulide

Ceyda Tuba SENGEL-TÜRK [1] *, Bilge BAYRAM [2]

[1] Department of Pharmaceutical Technology, Faculty of Pharmacy, Ankara University, Anadolu, 06100, Ankara, Turkey.
[2] Tokat State Hospital, Tokat, Turkey.
* Corresponding Author. E-mail: ctsengel@pharmacy.ankara.edu.tr (C.T.S.T.); Tel. +90-312-203 31 52.

Received: 06 May 2021 / Revised: 23 May 2021 / Accepted: 17 June 2021

ABSTRACT: The aim of this study is to develop PLGA based nanoparticle drug delivery systems loaded with nimesulide which is a non-steroidal anti-inflammatory (NSAI) agent and a selective COX-2 inhibitor that proved its antineoplastic and antitumor activity on cancer carsinogenesis and chemotherapy via epidemiological, pathological, clinical and biological studies in the last years, and modify their surface properties using chitosan chloride. For this purpose, Nimesulide loaded PLGA nanoparticles modified with chitosan chloride were developed with solvent evaporation-diffusion-salting out method. Nanoparticle formulations were evaluated for production yield, process efficacy, particle size and polydispersity index (PDI), surface charge, morphologic properties, redispersibility index, residual PVA amount and *in vitro* dissolution rate profiles. The results obtained showed that surface modification with chitosan chloride was significantly effective statistically on the final properties of the nanoparticles. With this study, it was shown that PVA and chitosan chloride combined can play a key role in developing cationic and positively charged nanoparticles.

KEYWORDS: Nimesulide; cancer; chitosan chloride; PLGA based polymeric nanoparticles; surface modification.

1. INTRODUCTION

Latest research on cancer ethiology which is the leading reason for mortality in the world, has documented that various animal and human tissues contain high concentrations of prostaglandin and genetic evidence has shown that cyclooxygenase (COX), especially COX-2 plays an important role in carsinogenesis. Epidemiological studies have reported an 85% increase in COX-2 levels especially in adenocarsinomas, and a decrease of 40-50% in mortality risk for various cancer types following long-term COX-2 inhibitor use. Nimesulide, which is a non-steroidal anti-inflammatory drug, is categorized as a selective COX-2 inhibitor. Chemotherapeutic effect of Nimesulid on various cancer types is independent from its COX inhibitor profile and the potential mechanism of its action is the induction of apoptosis and the inhibition of cell proliferation [1-3].

Anti-cancer agents developed as nanoparticle drug delivery systems are an important part of drug delivery research. Increasing the effect of antineoplastic agents in cancer treatment, reducing the frequency of adverse effects and increasing patient compliance by targeting the tumor tissue with nanoparticle drug delivery systems is deemed to be the most promising approach and is defined as the precursors of personalized chemotherapy [4]. The fundamental mechanism explaining the effect of nano sized carriers in cancer treatment is the high surface affinity between the tumor tissue epithelium, which has a high absorbance potential, and the nanoparticles. The level of affinity can be increased significantly by especially using cationic materials. The surface modification of the nanoparticles that are developed with cationic polymers in order to change the affinity to the epithelium is considered to be the most fundamental approach [5, 6]. Cell-based studies has proven that optimal coating of the nanoparticles with cationic polymers is shown to increase the contact period and therefore increase the transport of anticancer agents through the cell membrane. Based on this strategy, the most used cationic polymer is chitosan which is natural and biodegradable. Chitosan is the

How to cite this article: Sengel-Turk CT, Bayram B. Development and *in-vitro* evaluation of chitosan chloride decorated PLGA based polymeric nanoparticles of nimesulide. J Res Pharm. 2021; 25(4): 379-387.

ISSN: 2630-6344

https://dx.doi.org/10.29228/jrp.28

most preferred polymer for formulating antineoplastic nanosized drug delivery systems, not just because of its positive charge, but also its mucoadhesiveness, biocompatibility, biodegradability and its ability to open the tight junctions between epithelial cells [7-9].

In this study, the effect of surface modification via chitosan chloride on the physicochemical properties of PLGA based nanoparticles was examined. For this purpose, nanoparticle drug delivery systems loaded with nimesulide (of which anticancer effect was proven) and chitosan chloride (which was chosen as the surface modification agent) were developed, and the effect of the presence and amount of chitosan chloride on the physicochemical properties of the nanoparticles were evaluated. Formulations were prepared with a novel technique that combines emulsion-diffusion-salting out and emulsion-solvent evaporation methods [10]. Poly (D,L-lactide-co-glycolide) acid (PLGA) (50:50 copolymer ratio) was chosen as the polymer and poly (vinyl alcohol) (PVA) was chosen as the emulsifier. Surface coating of the nanoparticles was achieved during the preparation of the formulations by in-situ addition.

2. RESULTS AND DISCUSSION

In this study, surface modification of PLGA nanoparticles was achieved with chitosan chloride using the emulsion-solvent evaporation-diffusion-salting out method, and its effect on the physicochemical properties of the nanoparticles were examined. In the formulations that had chitosan chloride as a coating agent, nanoparticles were successfully formed. When PVA was taken out of the formulation process, it was seen that chitosan chloride was not effective enough to reduce the interface tension, therefore leading to no nanoparticle formation.

The production yield and encapsulation efficiency of the nanoparticle formulations produced using the emulsion-solvent evaporation-diffusion-salting out method are given in Table 1. The production yield was found to be between 88.73–90.16%, and it was observed that the effect of the addition of chitosan chloride was not statistically significant ($p>0.05$). When the production yields of the formulations were compared, it was determined that the addition and amount of chitosan chloride played an important role on the encapsulation of nimesulide, yielding statistically significant results ($p<0.05$). It was seen that when the amount of chitosan chloride added in the formulation with PVA was increased, the encapsulation efficiency of the active substance decreased. It is thought that this is observed due to the fact that chitosan chloride is not only on the surface of the nanoparticles but also partially with nimesulide and loaded in the matrix structure due to the use of an in-situ coating method for surface modification in particular; and the more chitosan chloride is used, the less nimesulide can be loaded into the nanoparticles (Table 1) (10, 11).

Table 1. Data of production yield and encapsulation efficiency.

Formulation code	Production Yield (% ± SD[a])	Encapsulation Efficiency (% ± SD[a])
PN1	90.16 ± 0.47	81.24 ± 0.49
PN2	89.46 ± 0.86	75.54 ± 0.76
PN3	88.73 ± 0.65	63.71 ± 0.44

[a] Standard deviation

Particle size and PDI values of the nanoparticles were given in Figure 1. The particle size distribution for nanoparticle delivery systems is expressed with PDI values. PDI is definded with the log-normal particle size distribution of the nanoparticles (12, 13). In our study, when the particle size data were examined, depending on the addition and the amount of the chitosan chloride the change in nanoparticle size data was shown to be statistically significant ($p<0.05$). In the case of chitosan chloride being in the aqueous phase with PVA, it was thought that chitosan chloride increased the surfactant properties of PVA, therefore resulting in smaller nanoparticles. When the chitosan chloride concentration was increased from 0.3 mg/mL to 0.6 mg/mL, it was observed that nanoparticle size increased significantly with the increasing viscosity ($p<0.05$). This increase in the size depending on the increasing chitosan chloride amount was also supported by literature (14). Particle size distribution values obtained were in a narrow range for all three formulations (PDI<0.2).

Zeta potential is an important parameter for the stability of the nanoparticle dispersion and particle mucoadhesion. High values whether negative or positive show ideal stability of the nanoparticle system, and the high charge provides protection from the aggregation by creating a strong repulsion between nanoparticles (14, 15). The zeta potential values are given in Figure 1. Zeta potential value of PN1 that did not contain chitosan chloride was determined to be -21.04 mV. The negative surface charge was caused by the ionized

carboxyl groups of PVA on the nanoparticle surface (16). In this study, in which we aimed to design and develop nimesulide loaded nanoparticle drug delivery systems for cancer treatment, chitosan chloride (which is cationic) was used in order to develop such nanoparticles that have different physicochemical properties, chitosan chloride and nanoparticle formulations with positive surface charge were developed. Surface charge of nanoparticles is an important parameter that affects their distribution within the body and uptake by the cells. Because of mucus having neutral pH and being an anionic polyelectrolyte, anionic nanoparticles are modified to have positive surface charge with cationic surfactants and mucoadhesive polymers in order to increase the electrostatic affinity of cell membranes to nanoparticles and the pull between mucus and the nanoparticles. The contact time between epithelium cells and cationic nanoparticles developed with suitable methods increases, therefore increasing cellular uptake (15, 17, 18). The difference in surface charge of the formulations modified with chitosan chloride was statistically significant ($p<0.05$). The zeta potential of the formulation that did not contain chitosan chloride (-21.04 mV) was shown to increase for the PN2 and PN3 formulations that contained increasing amounts of chitosan chloride to +23.54 mV and +31.58 mV, respectively. The particle size increased with the addition of increasing amounts of chitosan chloride, yielding similar results with zeta potential data. In a study conducted by Nafee et al. similar results were obtained and it was reported that the increase in surface charge was found to be related to the increasing amount of chitosan chloride, because of the high amount of protonated amino groups of the chitosan molecule on the nanoparticle surface (14).

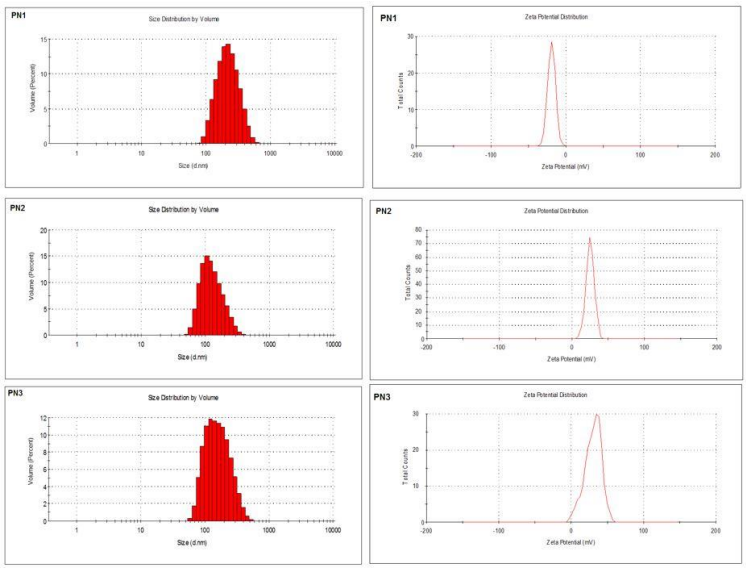

Figure 1. Particle size and zeta potential graphs of undecorated and chitosan chloride decorated PLGA nanoparticles.

The images obtained with TEM analysis showed that all three formulations could be developed to be remarkably spherical (Figure 2). An accumulation layer of chitosan chloride on the surface of the nanoparticles was seen during the TEM analysis. The images also confirmed the zeta potential findings, supported the particle size data, and showed that nanosized particles could be developed with the methods used in our study.

When the physicochemical charasteristics of PLGA which was used as the polymer was evaluated, it was found to hydrolyze in aqueous mediums. In order to maintain the chemical stability of the nanoparticles, it was advised to turn the nanoparticles into solid form with methods such as freeze drying (19). Nanoparticles developed and stored after lyophilization are evaluated for stability change with redispersibility index analysis. With this analysis, it is determined whether the particles form aggregates after the lyophilization process and whether the lyophilized particles can be successfully separated from each other when dispersed in any desired medium. The redispersibility index values are given in Table 2. Because the redispersibility index is a ratio of average size of redispersed nanoparticles to average size of formulated nanoparticles, low values we obtained mean that the nanoparticles have high redispersibility properties. When the data for our formulations was evaluated, all redispersibility values were found to be above 1.00 (in a range of 1.0201 – 1.5524). When compared with literature, it was seen that all formulations showed optimal redispersibility properties (20, 21).

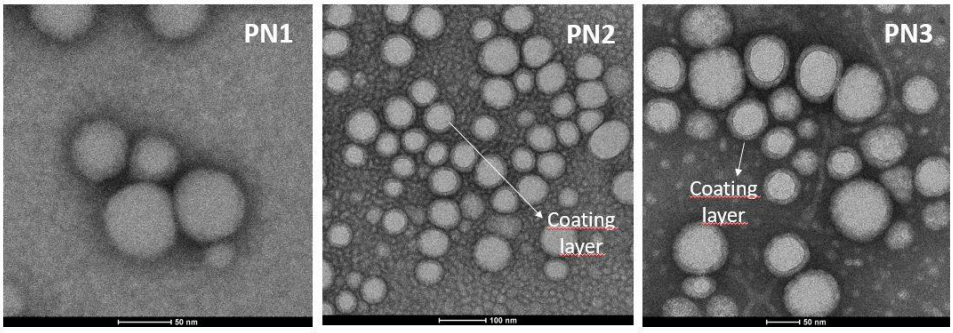

Figure 2. TEM images of undecorated and chitosan chloride decorated PLGA nanoparticles.

Table 2. Redispersibility index values of nanoparticles.

Formulation code	Lyophilized nanoparticle size (nm ± SD[a])	Original nanoparticle size (nm ± SD[a])	Redispersibility Index
PN1	221.58 ± 2.66	217.20 ± 4.46	1.0201
PN2	137.30 ± 1.24	111.90 ± 1.21	1.2269
PN3	173.56 ± 2.80	111.80 ± 0.49	1.5524

[a] Standard deviation

The addition of a surfactant agent is required in the formulation process of nanoparticle delivery systems regardless of the method preferred. Despite PVA being the most used agent because of its small molecule size and being able to provide narrow particle size distribution for nanoparticles, it is known to leave residue on particle surfaces and form a web-like structure with the polymer, therefore making its removal difficult. Strong interactions occur between PVA and PLGA at the water/dichloromethane interface, resulting in PVA binding irreversibly to the particle surface. Binding mechanism of PVA is thought to occur via PLGA-PVA molecule interpenetration by removal of the organic phase from the interface during nanoparticle formation. For this reason, washing process in the ultracentrifugation step during the formulation process with PVA becomes significant. PVA that couldn't be removed with this process was found to be higher than 13% (w/w), affecting physical properties and cell membrane interactions of the nanoparticles (16, 22). For this reason, residual PVA amount on surfaces of nanoparticles that are formulated with PVA are given in Table 3. The residual PVA percentage for PN1 which did not contain chitosan chloride was 18.73%, while PN2 and PN3 which contained various amounts of chitosan chloride were 5.66% and 1.24% respectively. This proves that chitosan chloride added with PVA was able to significantly reduce the formation of the web-like structure between PVA and PLGA, and the binding of PVA to the nanoparticle surface.

Table 3. Residual amount of PVA on the surface of nanoparticle formulations.

Fomulation code	Residual PVA amount (% ± SD[a])
PN1	18.73 ± 0.47
PN2	5.66 ± 0.79
PN3	1.24 ± 0.64

[a] Standard deviation

The in-vitro release profiles of our studies are given in Figure 3. Differences between the release profile obtained were found to be statistically significant in terms of presence and amount of chitosan chloride ($p<0.05$). The release rate of active substance in formulations containing chitosan chloride was shown to increase ($p<0.05$). When chitosan chloride concentration was increased from 0.3 to 0.6 mg/mL, the release rate was significantly reduced depending on the increasing particle size. The increase in the release rate of the active substance from the PLGA nanoparticle formulations is an expected result in terms of the increased solubility of chitosan chloride at alkaline pH values compared to unmodified chitosan, depending on the deacetylation degree of chitosan chloride (23). It is thought that the increase in the release rate profiles of nanoparticle formulations coated with chitosan chloride is due to both the structure of the modified chitosan in the form of chloride salt and the small particle sizes of the nanoparticle formulations, which were determined as 137.30 nm (PN2) and 173.56 nm (PN3).

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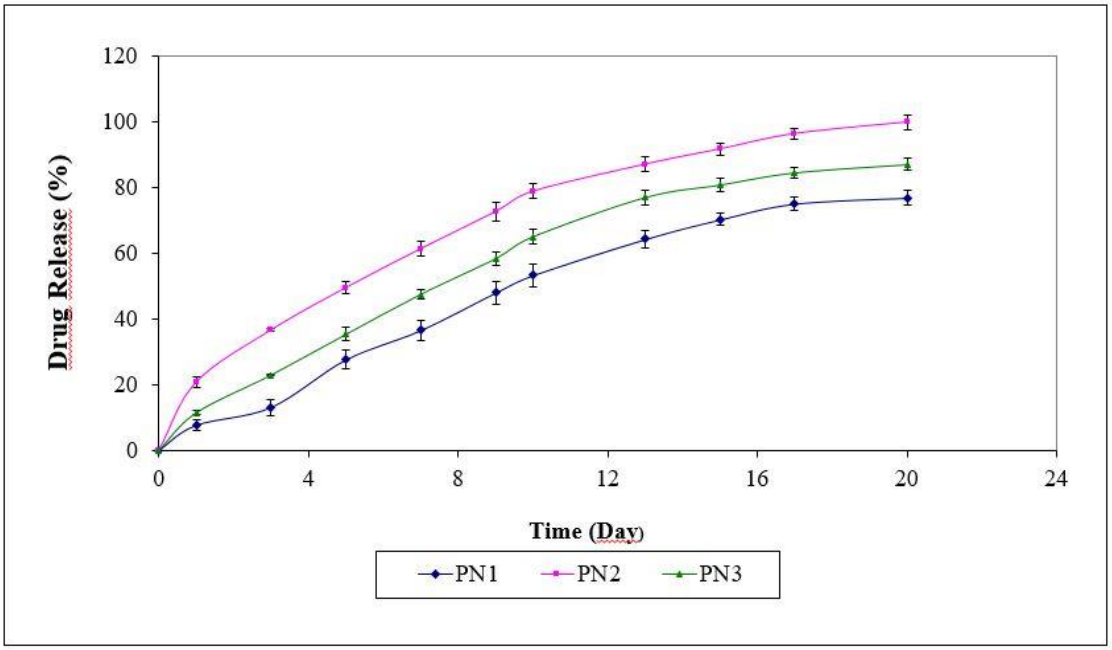

Figure 3. In-vitro release profiles of nimesulide from undecorated and chitosan chloride decorated PLGA nanoparticles.

Storage stability of the developed polymeric nanoparticles were also evaluated at 5±3 °C for three months. Encapsulation efficiency, particle size, PDI and zeta potential values of the nanoparticles were examined within this scope. Statistical evaluation of the developed nimesulide encapsulated undecorated and chitosan chloride decorated PLGA nanoparticles indicated that the encapsulation efficiency, particle size, PDI, and zeta potential values of nano-sized particles showed no significant change, and also indicated excellent chemical stability for encapsulated nimesulide over a three months period ($p > 0.05$) (Table 4).

Table 4. Stability data of undecorated and chitosan chloride decorated PLGA nanoparticles at 5±3 °C.

Formulation code	Storage time	Encapsulation efficiency (% ± SD[a])	Particle size (nm ± SD[a])	PDI (Mean ± SD[a])	Zeta potential (mV ± SD[a])
PN1	Initial	81.24 ± 0.49	221.58 ± 2.66	0.092 ± 0.02	-21.04 ± 1.08
	3 months	79.27 ± 2.54	226.45 ± 4.72	0.106 ± 0.11	-19.71 ± 2.44
PN2	Initial	75.54 ± 0.76	137.30 ± 1.24	0.115 ± 0.01	+23.54 ± 1.38
	3 months	76.43 ± 3.47	141.18 ± 4.86	0.120 ± 0.09	+25.83 ± 3.01
PN3	Initial	63.71 ± 0.44	173.56 ± 2.80	0.172 ± 0.01	+31.58 ± 2.28
	3 months	65.26 ± 2.49	179.69 ± 5.81	0.163 ± 0.08	+32.29 ± 3.11

[a] Standard deviation

3. CONLUSION

In this study, the preparation of PLGA-based nanoparticle formulations of nimesulide, an anti-inflammatory agent that proved its efficacy as an antiproliferative agent and use in the treatment of various cancer types, and the preparation of these nanoparticle formulations developed with an in-situ surface modification method using chitosan chloride for the first time. With this research, it was determined that, when used without chitosan chloride, PVA which is an emulsifying agent in the formulation could not deliver the cationic charge needed to ensure a high level of interaction with negatively charged cancer cells. On the other hand, if only chitosan chloride is used without PVA, it has been determined that the stabilization of the particles that will provide nanoparticle formation cannot be achieved, thus the formation of nanoparticles does not occur. As a result, it has been determined that the combined use of chitosan chloride and PVA in the design of nanoparticle formulations is a key point in the design of cationic and positively charged PLGA nanoparticles and that the physicochemical properties of PLGA nanoparticles can be modified using appropriate amounts of chitosan chloride.

https://dx.doi.org/10.29228/jrp.28
J Res Pharm 2021; 25(4): 379-387

4. MATERIALS AND METHODS

4.1. Materials

The materials used were Nimesulide (Ulkar Chemicals Company, Istanbul, Turkey), poly (D,L-lactic-co-glycolic acid (PLGA, lactic to glycolic acid ratio 50:50, molecular weight 40-75 kDa), polyvinyl alcohol (PVA, MA 9-10 kDa) (Sigma-Aldrich, Steinheim, Germany), chitosan chloride (Protosan UP CL 113, Molecular weight <150 kDa, deacetylation degree 75-90%, FMC Bioploymers, Norway), magnesium chloride hexahydrate (Merck Chem. Co., Darmstadt, Germany), purified water (Milli-Q® Plus System (Millipore Corp., Molsheim, France). All other chemicals used were at least of reagent grade.

4.2. Preparation of undecorated and chitosan chloride decorated PLGA nanoparticles

In this study, the purpose is to develop nanoparticle formulations with PLGA and to examine the effect of chitosan chloride added to the surfactant in varying concentrations on the physicochemical properties of the nanoparticles. This method is used for modification of the surface properties of nanoparticles and is defined as the in-situ coating method [10]. In our study, the emulsion-solvent evaporation-diffusion-salting out method which is a combined preparation method that allows the surface modification in developing nimesulide loaded polymeric nanoparticles [24], was used. For this purpose, dichloromethane and acetone were used as organic phase, PVA was used as surfactant, and $MgCl_2.6H_2O$ as salting-out agent. Nimesulide was dissolved in 2 mL of dichloromethane and polymer was dissolved in 6 mL of acetone in a 1:10 ratio and mixtures were combined. This organic phase mixture was added into the aqueous phase containing a surfactant agent (PVA-2.5% w/v) and a salting-out agent (magnesium chloride-$MgCl_2.6H_2O$-45% w/v) and sonicated with an ultrasonic homogenizer (Bandelin Sonopuls HD 2070, Bandelin Elec., Germany) with a power of 55 watts, in an ice bath for 5 minutes. A second aqueous phase containing 2.5% w/v of PVA has been added to the resulting dispersion and the organic solvent was evaporated by stirring the mixture on a magnetic stirrer for 24 hours. Resulting nanoparticles were collected by centrifuge in a high speed centrifuge (Sigma Laboratory Centrifuge 3KS30, Germany) with 25.000 rpm for an hour and washed with ultrapure water in order to remove impurities. Resulting dispersion was frozen at a temperature of -80°C for 24 hours then lyophilized (Christ Gamma 2-16 LSC, Martin Christ Gef., Germany) for 48 hours. In this study, to examine the effect of chitosan chloride as surface modification agent, it has been added to the PVA solutions during the formulation process. The contents of the formulations developed for this purpose are given in Table 5.

Table 5. Compositions of uncoated and chitosan chloride-coated PLGA nanoparticles.

Formulation code	Content of the Formulations		
	Polymer	Surfactant	Coated Agent and Amount
PN1	PLGA	PVA	-
PN2	PLGA	PVA	Chitosan chloride (0.3 mg/ml)
PN3	PLGA	PVA	Chitosan chloride (0.6 mg/ml)
PN4[b]	PLGA	-	Chitosan chloride (0.3 mg/ml)
PN5[b]	PLGA	-	Chitosan chloride (0.6 mg/ml)

[b] No nanoparticles were formed.

4.3. Physicochemical characterization of the nanoparticles

4.3.1. Production yield

To evaluate production yield which is one of the important parameters in nanoparticle characterization, nanoparticles were weighed to get the solid nanoparticle amount, and this value and the solid material amount were used to calculate the production yield in percentage (25, 26) (Eq.1).

Production Yield (%) = (Total nanoparticle amount/Total solid material amount) x 100 (Eq.1)

4.3.2. Encapsulation efficiency

To determine the amount of active substance in the nanoparticles, a known amount of nanoparticles was weighed, dissolved in dichloromethane and sonicated with ultrasonic homogenizer with a power of 30 watts for 10 minutes. Dissolved nanoparticles were diluted to various concentrations and the nimesulide amount was determined with UV spectrophotometric method at 297 nm. In determining drug loading

capacity (DLC) (Eq. 2), and encapsulation efficiency percentages of the nanoparticles, the equations below were used (n=3) (Eq. 3) (27).

DLC (%) = (Amount of drug in nanoparticles/Amount of nanoparticles) x 100 (Eq. 2)

Encapsulation Efficiency (%) = (Actual drug loading % / Theoretical drug loading %) x 100 (Eq. 3)

4.3.3. Particle size, PDI and surface charge measurements

Particle size, PDI value and surface charge were determined with photon correlation spectroscopy (Zetasizer Nano Series, Nano ZS, Malvern Inst., Worcestershire, UK). For this purpose, 2 mg of nanoparticles were suspended in 20 mL of ultrapure water and sonicated in ultrasonic bath for 15 minutes. Particle size and PDI measurements were repeated five times for each formulation. For zeta potential measurements, nanoparticle formulation dispersions in ultrapure water were put in a disposable, 1 mL capacity zeta potential measurement cell, and the measurements were repeated five times. All the measurements were performed with the device set to room temperature.

4.3.4. Morphological characteristics of nanoparticles

Transmission Electron Microscopy (TEM) analysis was used in order to successfully see the morphology of the nanoparticles and to determine the presence of chitosan chloride layer on the surface of the nanoparticles. Lyophilized nanoparticles were diluted in bidistilled water in various amounts and sonicated in ultrasonic bath for 15 minutes. 10 µl's of nanoparticle dispersion was dropped on a carbon film covered with copper and was dried in a lidded petri dish at room temperature overnight. The samples were dyed with 2% w/v uranyl acetate solution before the imaging and the residual solution was removed by blotting with a filter paper. Electron micrograph of said samples were acquired in TEM (FEI Tecnai G2 Spirit Biotwin TEM, ABD).

4.3.5. Redispersibility index

In order to determine the redispersibility index of the nanoparticles, the particle sizes were determined with photon correlation spectroscopy before the lyophilization using the nanoparticle dispersion during the formulation process, and after the nanoparticles were lyophilized. Lyophilized nanoparticles were redispersed in ultrapure water and the measurements were repeated. Redispersibility index values of the formulations were calculated with the equation below (Eq. 4) (19).

Redispersibility index = (Lyophilized nanoparticle size / Original nanoparticle size) (Eq. 4)

4.3.6. Residual amount of PVA

In order to determine the amount of PVA that was not removed by washing or was not attached to the surface because of the modification process made with chitosan chloride, a colorimetric method based on the determination of a colored complex formed between PVA's two hydroxyl groups and iodine molecule was used. For this purpose, 2 mg of lyophilized nanoparticle were dispersed in 2 mL of 0.5 M NaOH and incubated at 60°C for 15 minutes. This causes PLGA that was used as the polymer to hydrolize. After the hydrolization process, the dispersion was neutralized with 900 µl 1 N HCl and the volume was completed to 5 mL with ultrapure water. To each solution, 3 mL of 0.65 M boric acid, 0.5 mL of 0.05 M/0.13 M I_2/KI solution and 1.5 mL of ultrapure water were added. The solutions were left to form the colored complex for 15 minutes at room temperature, and the amount of PVA that was not removed by washing or that was not attached to the surface because of the surface modification process made with chitosan chloride was determined from the absorbance of the colored complex with UV spectrophotometry at 690 nm (n=3) (24).

4.3.7. In vitro drug release studies

Static method was chosen for *in vitro* release studies of the formulations based on the literature (20). A known amount of nanoparticles were put in dialysis membrane bags (molecular weight cut-off: MWCO = 12,000 Da) submerged in lidded jars containing 100 mL of pH 6.8 phosphate buffer and were put in an

incubated shaker bath with a speed of 50 rpm at 37°C. At pre-determined time intervals, known amount of aliquots were collected, analyzed with UV spectroscopy at 385 nm, and the concentration of active substance was calculated from the absorbances. Aliquot amount of fresh medium at the same temperature was added to the existing release medium (n=3).

4.3.8. Storage stability

The storage stability of the nimesulide encapsulated polymeric nanoparticle formulations was also evaluated. For this purpose, the lyophilized nanoparticles were stored at 5±3 °C climatic condition for three months (28). Encapsulation efficiency, particle size, PDI, and zeta potential values were examined (n = 3).

Author contributions: Concept – C.T.S.T.; Design –C.T.S.T.; Supervision – C.T.S.T.; Resources – C.T.S.T.; Materials – B.B.; Data Collection and/or Processing – B.B., C.T.S.T.; Analysis and/or Interpretation – B.B., C.T.S.T.; Literature Search – B.B., C.T.S.T.; Writing – C.T.S.T.; Critical Reviews – C.T.S.T., B.B.

Conflict of interest statement: The authors declared no conflict of interest

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