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Surface modified zeolite-based granulates for the sustained release of diclofenac sodium

Carla Serri [a], Bruno de Gennaro [b], Vincenzo Quagliariello [c], Rosario Vincenzo Iaffaioli [c], Giuseppe De Rosa [d], Lilia Catalanotti [b], Marco Biondi [d,e,*], Laura Mayol [d,e]

[a] Dipartimento di Scienze del Farmaco e Prodotti per la Salute, Università di Messina, Piazza Pugliatti 1, Messina, Italy
[b] Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università di Napoli Federico II, Piazzale V. Tecchio 80, 80125 Napoli, Italy
[c] Department of Abdominal Oncology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Fondazione "G. Pascale"-IRCCS, Naples, Italy
[d] Dipartimento di Farmacia, Università di Napoli Federico II, Via D. Montesano 49, Napoli, Italy
[e] Interdisciplinary Research Centre on Biomaterials – CRIB – Università di Napoli Federico II, P.le Tecchio, 80, Napoli, Italy

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ABSTRACT

In this study, a granulate for the oral controlled delivery of diclofenac sodium (DS), an anionic sparingly soluble non-steroidal anti-inflammatory drug, has been realized by wet granulation, using a surface modified natural zeolite (SMNZ) as an excipient. The surface modification of the zeolite has been achieved by means of a cationic surfactant, so as to allow the loading of DS through ionic interaction and bestow a control over the drug release mechanism. The granules possessed a satisfactory dosage uniformity, a flowability suitable for an oral dosage form manufacturing, along with a sustained drug release up to 9 h, driven by both ion exchange and transport kinetics. Furthermore, the obtained granulate did not elicit a significant cytotoxicity and could also induce a prolonged anti-inflammatory effect on RAW264.7 cells. Taking also into account that natural zeolites are generally abundant and economic, SMNZ can be considered as an attracting alternative excipient for the production of granules with sustained release features.

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1. Introduction

In pharmaceutical dosage forms, the choice of excipients strongly influences the design and in vivo performance of the administered active molecule. In this context, polymers play a crucial role and can also be endowed with further properties, such as the sensitivity to specific stimuli, the ability to induce enzyme inhibition, the enhancement of intestinal epithelium penetration, the inhibition of efflux pump and taste masking (Karolewicz, 2016). The proper choice of polymeric excipients also allows to increase drug solubility/bioavailability, as for the case of cyclodextrins and/or amphiphilic copolymers (Miro et al., 2013; Conte et al., 2016), the improvement of active substance stability (Mayol et al., 2015) and therapeutic activity (Moyano-Mendez et al., 2014), along with and the possibility to target the drug to a specific site in the organism. Furthermore, mesoporous silica-based materials allow a high loading efficiency and tunable drug release profile taking advantage of their high surface density of hydroxyl groups, which can be easily functionalized (Shen et al., 2013). However, despite the promising skills of these materials, in vivo performances of silica-based materials such as biodistribution, acute and chronic toxicities, long-term stability, circulation properties and targeting efficacy are still to be investigated (Yang et al., 2012).

Further interesting materials employed in the manufacturing of pharmaceutical dosage forms are minerals, which are attracting a significant deal of interest due to their low cost and widespread use in the pharmaceutical industry, both as active molecules and as excipients (Carretero and Pozo, 2009; Carretero and Pozo, 2010). The used minerals must satisfy numerous biological and technological requirements. In particular, they must be non-toxic to humans and, furthermore, they must possess a great adsorption capacity and specific surface area, a significant swelling ability, along with suitable thixotropic and colloidal properties when used in semisolid preparations (Carretero and Pozo, 2010; Viseras et al., 2007). Generally, synthetic analogues of natural minerals are employed in the pharmaceutical industry since the synthesis process is usually less expensive compared to the extraction/purification of natural minerals. The use of natural minerals prevails in the case of abundant and inexpensive materials, or if the synthesis process is expensive and/or complicated.

Among natural minerals, some tectosilicates, such as zeolites, are being studied as components of pharmaceutical dosage forms due to their adsorption properties and ion exchange ability (Cerri et al., 2004; Bonferoni et al., 2007; Carretero and Pozo, 2009). Zeolites have been studied for their possible use as drug carriers, adjuvants in cancer

* Corresponding author at: Dipartimento di Farmacia, Università di Napoli Federico II, Via D. Montesano 49, Napoli, Italy.
E-mail address: mabiondi@unina.it (M. Biondi).

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therapy, and antimicrobial agents (Danilczuk et al., 2008). Actually, the pharmaceutical industry is showing an increasing interest in the use of zeolitic materials as substrates for the release of ionic drugs due to their well-known ion exchange ability. In particular, zeolites possess a porous structure, characterized by channels and cavities in the molecular scale, that allow the transport of molecules within the crystal towards the external environment and vice versa (Armbruster and Gunter, 2001). Micronized zeolites can be used as carriers of active molecules for oral or topical administration, because of their great specific surface area and consequent ability to adsorb large amounts of ions (Rivera and Farias, 2005). Among zeolites, clinoptilolite has been attracting an increasing interest in the pharmaceutical field as a possible excipient in pharmaceutical dosage forms for sustained drug release. For example, clinoptilolite has been successfully employed as a carrier for zinc and erythromycin for topical utilization (Cerri et al., 2004; Bonferoni et al., 2007). Indeed, clinoptilolite possesses many interesting properties: for example, it is endowed with significant antimicrobial, antitumour and antiviral activity (Pavelic et al., 2001; Grce and Pavelic, 2005; Katic et al., 2006) and has been employed to treat skin wounds, in renal dialysis to remove ammonium ions from body fluids and also as an antidiarrheal or antiacid agent (Rodriguez-Fuentes et al., 1997; Mumpton, 1999; Rodriguez-Fuentes et al., 2006). Furthermore, clinoptilolite is biologically inert (Adamis et al., 2000), which makes it particularly attractive for pharmaceutical applications.

In its native state, clinoptilolite is a cationic exchanger and bears superficial anions which, in an aqueous suspension, are located within zeolite channels and cavities together with water molecules. Therefore, to promote the adsorption of anionic drug molecules, clinoptilolite surface can be modified with cationic surfactants, which can reversibly remove or replace water molecules. The adsorption of a cationic surfactant is driven by ion exchange coupled to hydrophobic interactions among the chains of the surfactant. Once at the surface of clinoptilolite, surfactant molecules replace superficial inorganic cations so as to saturate its external cation exchange capacity (ECEC); further surfactant addition leads to the formation of a cationic molecular bilayer which, in turn, causes the inversion of surface charge. In particular, this surface modification allows retaining anions, while repelling cations and enhancing the partitioning of neutral species in the hydrophobic burden of the cationic surfactant bilayer (Li and Bowman, 1998). It must also be underlined that the adsorption of surface active agents at the solid-liquid interface leads to an increase of clinoptilolite hydrophobicity, and therefore a promoted affinity for many drug molecules.

In two recent studies (de Gennaro et al., 2015; Serri et al., 2016), clinoptilolite-rich rock from California (CLI_CA) has been selected over two other zeolites as a suitable carrier of drugs. The surface of micronized CLI_CA has been modified with cetylpyridinium chloride (CP), a cationic surfactant, to facilitate the loading of anionic diclofenac sodium (DS). The obtained zeolite particles could sustain the release of DS in simulated intestinal fluid (SIF) for at least 5 h. Despite the promising results, the samples did not meet the Pharmacopoeia requirements with regard to flow properties (de Gennaro et al., 2015). In order to overcome this issue, the objective of this work was to formulate a granulate containing clinoptilolite, intended for the oral administration and sustained release of DS. To this aim, CLI_CA superficially modified with CP has been used to prepare granules using cornstarch as a glidant/disintegrating agent and hydroxypropyl methylcellulose (HPMC) as a release rate controlling/binder excipient. The obtained granules have been evaluated for their flowability, drug content/release kinetics, and cellular outcome in terms of cytotoxicity and anti-inflammatory response.

2. Materials and methods

2.1. Materials

A clinoptilolite-rich rock from California (CLI_CA; St. Cloud Mining Company, CA, USA) was used. A thorough characterization of these materials has been recently carried out by Cappelletti et al., on samples ground to a particle size suitable for oral administration (Cappelletti et al., 2015). XRPD quantitative mineralogical analyses, XRF chemical analyses, SEM-EDS investigations were carried out to identify their chemical and mineralogical features whose technological specifications are reported in Table 1. Diclofenac sodium (DS) (CAS [15307-79-6]), starch, hydroxypropyl methylcellulose (HPMC), magnesium stearate and talc were from Farmalabor (Italy). The surfactant cetylpyridinium-chloride (CP-Cl) (99.0–102.0%, CAS [6004-24-6]), hydrochloric acid (HCl), and the salts needed for the preparation of the simulated intestinal fluid (SIF) were obtained from Sigma-Aldrich (USA).

2.2. Preparation of surface-modified natural zeolites (SMNZ)

Before the technological and cellular studies, potentially unsafe cations were removed from the zeolite by washing with a 1 M solution of NaCl as previously described (de Gennaro et al., 2015,de Gennaro et al., 2016). The obtained Na^+-rich samples were then washed with distilled water and air-dried. Afterwards, the surface modification with the cationic surfactant was performed by vigorous mixing the CLI_CA rock samples with an aqueous solution of CP-Cl (Cappelletti et al., 2015) and the loading of DS on SMNZ was carried out as indicated in a previous work (de Gennaro et al., 2015). Finally, once liquid and solid were separated by filtration and the liquid spectrophotometrically analyzed (UV-1800, Shimadzu Laboratory World, Japan; $\lambda = 275$ nm), to assess the DS content loaded into SMNZ.

2.3. Preparation of the granules

Wet granulation is a technique which allows to promote the coalescence of primary particles by means of a binder liquid. This helps to improve the flowability of the powder and, consequently, to favor the uniformity of drug content in the dosage form. In this work, prior to granulate preparation, the surface-modified natural zeolite (SMNZ) powder was sieved with a 250 μm mesh. Then, the granules containing bare or DS-loaded SMNZ were prepared by mixing 5.0 g of SMNZ, 3.5 g of HMPC and 7.2 g of starch, through the wet granulation technique, performed by progressively adding water (approximately 12 ml) to the dry powder mixture and accurately kneading the wet mass with a pestle within a porcelain mortar until a homogeneous smooth mass was obtained. The latter was then forced through a mesh (2000 μm size) and the collected granules were dried for 2 h at 40 °C. After drying, the dry granules were forced through a mesh (1000 μm size) by a pestle and, finally, talc (200 mg) and magnesium stearate (100 mg) were added as lubricants.

2.4. Characterization of the granules

The prepared granules were evaluated for their size, surface area, drug content uniformity and Carr's Index, indicated as CI(%). The size of the granules was determined by sieving, following the indications of the European Pharmacopoeia (8th Ed). Briefly, a known mass of the granules was fractionated using four sieves (mesh opening 2000, 1000, 500, 300 μm) and the size distribution was obtained from the relative amounts of granules, gravimetrically quantified. We have also calculated the median equivalent diameter, circularity and aspect ratio of

Table 1
Technological features of the investigated materials.

	CLI_CA
CEC experimental (meq/g)	1.97
ECEC (meq/g)[a]	0.49
Zeolite content (wt.%)	80.20

[a] Experimental data obtained using CP-Cl surfactant.

the obtained granules through image analysis assisted by ImageJ software (v. 1.41).

The surface area of the DS-loaded granules was evaluated by Micromeritix ASAP 2020 porosimeter following a procedure previously described (Cappelletti et al., 2015).

The DS-loaded granules were tested for their drug content uniformity, according to the indications of European Pharmacopoeia. Briefly, ten aliquots of the granulate (around 50 mg) were poured in 100 ml of distilled water at room temperature and stirred for 6 h to allow the complete solubilization of the drug; thereafter, 5 ml of the obtained solution were centrifuged and the supernatant was filtered through a 0.2-μm membrane filter. Subsequently, after suitable dilution, the drug content was quantified by spectrofluorimetric analysis ($\lambda = 275$ nm). The absorbance was linearly dependent on DS concentration in the 0.2–10 μg/ml range; r^2 was higher than 0.99.

Granulate flowability was evaluated by the Carr's index (CI), the Hausner ratio (HR) and the angle of repose (α_R). Carr's index was obtained following a procedure already described in two previous articles (de Gennaro et al., 2015; Serri et al., 2016). Hausner ratio was calculated by the following equation:

$$HR = \frac{\rho_T}{\rho_0}, \tag{1}$$

where ρ_T is the tapped density, i.e. the mass-to-volume ratio of the granulate after 1250 taps of the sample (using an automatic tapping machine, as described in European Pharmacopoeia), and ρ_0 the bulk density, i.e. the mass-to-volume ratio of the granulate before compaction. The angle of repose was determined by pouring 50 g of the granulate through a stainless steel funnel (bottom diameter: 1 cm, height 3.4 cm) and, subsequently, evaluating the conical pile formed on the platform underneath the funnel. The angle of repose was calculated by the following equation:

$$\alpha_R = \tan^{-1}\left(\frac{2h}{D-d}\right) \tag{2}$$

where h is the height of the funnel with respect to the underlying platform, D the diameter of the base of the cone and d the diameter of the funnel bottom.

Granule hygroscopicity has been measured by a climatic cell equipment (MSL EC125). For each experiment, the samples (approximately 10 g) were loaded in a humidity chamber at room temperature with an initial zero relative humidity (RH). Subsequently, RH values have been set to 0.3, 0.5, 0.75 and 0.9. Each RH value was maintained until the mass of the sample reached a plateau value. Surface area was obtained by the Brunauer–Emmett–Teller (BET) approach, using nitrogen microporosimeter (Micromeritics ASAP 2020), as described in a previous paper (Cappelletti et al., 2015).

2.5. In vitro dissolution/drug release tests

The dissolution/release studies of DS from granulate were carried out at 37 °C under mild agitation (100 rpm) using the Sotax dissolution rate test apparatus type II model (USP Apparatus 1; SOTAX, Switzerland). For each experiment, samples of the granulate (1 g) were placed into 900 ml of simulated gastric fluid (SGF; 2.0 g NaCl, 7 ml HCl and distilled water to a final 1000 ml volume; pH = 1.2) for 2 h and, thereafter, in simulated intestinal fluid (SIF, 68.05 g KH_2PO_4 and 8.96 g NaOH in 10 l of water; pH = 6.8) for further 10 h. At predetermined time points, 5 ml of the release medium were withdrawn and replaced with an equal volume of fresh medium. To quantify the released DS, samples were filtered through a 0.2 μm membrane-filter and the drug in the supernatant was quantified by spectrophotometric analysis as described in the *Preparation of surface-modified natural zeolites (SMNZ)* section. As a control, the same experiments were carried out on bare SMNZ, without granulation. During DS release studies, the dissolution of the granulate was kept under observation.

DS dissolution was further characterized by the dissolution efficiency (DE), which was calculated based on Khan's work (Khan, 1975). Briefly, DE was obtained by the following equation:

$$DE = \frac{\int_{t1}^{t2} y \cdot dt}{y_{100} \cdot (t_2 - t_1)} \times 100, \tag{3}$$

where y is the percentage of dissolved/released DS. Eq. (3) is the ratio between the area under the dissolution profile from t_1 to t_2 time points and the maximum dissolved percentage in the same time period.

2.6. Cell viability

The cytotoxicity of free DS, placebo granulate, and DS-loaded granulate against mouse macrophages (RAW264.7 cell line) was screened by measuring the activity of mitochondrial dehydrogenase through a modified MTT assay (Dojindo Molecular Technologies Inc., Rockville, MD). Macrophages were maintained in 96-well polystyrene plates (10^4 cells/well in Dulbecco's Modified Eagle's medium (DMEM) medium (Gibco®)) containing 10% v/v FBS (Fetal Bovine Serum) and 1% w/v penicillin/streptomycin (Pen-Strep), and placed at in a humidified incubator (37 °C, 5% CO_2 atmosphere). After 24 h of growth, cells were washed with PBS. Subsequently, the cytotoxic effect of: (i) DS-free CLI_CA granulate (0.05–5 mg/ml concentration range); (ii) free DS (0.0075–0.078 mM concentration range); (iii) drug-loaded CLI_CA granulate against RAW264.7 cells has been verified. The drug and the granulate were dissolved/suspended in full culture medium in all cases. Subsequently, cells were incubated for 24 h washed three times with PBS (pH = 7.4) and further incubated with 100 μl of an MTT solution in cell culture medium (0.5 mg/ml) for 4 h at 37 °C. The absorbance was measured at 450 nm with the Tecan Infinite M200 plate-reader with an I-control software. The cell viability (%) was finally obtained from the following equation:

$$\text{Cell viability}\,(\%) = 100 \times \frac{[A]_{test}}{[A]_{control}}, \tag{4}$$

where $[A]_{test}$ and $[A]_{control}$ are the absorbance of the test sample and of the control cells seeded in the presence of cell culture medium only, respectively. Then, the protein content was measured with the Thermo Scientific Micro BCA™ protein assay kit (Pierce). Briefly, cells were washed with cold PBS and incubated with 150 μl of cell lysis buffer (15 min, 0.5% v/v Triton X-100 in PBS). Finally, 150 μl of Micro BCA protein assay kit reagent were added and the absorbance of the resulting solution was measured at 562 nm. The cytotoxicity measurements were normalized with respect to the total protein content.

2.7. Measurement of cytokines

Cytokine production in cell culture supernatant was determined by Bio-Plex Pro mouse Cytokine Chemokine, and Growth Factor Plex Panel kit as reported elsewhere (You et al., 2013); Briefly, RAW264.7 cells were seeded in 12-wells plate (1.2×10^5 cells/well) with DMEM and incubated as described in the *Cell viability* section. The cells were pre-incubated for 24 h and starved in serum-free medium for 2.5 h and, afterwards, were treated for 1 h, 2 h, 4 h and 6 h with or without 0.1 ml of free DS or DS-loaded zeolite at an overall 0.1 or 0.2 mM concentration of the drug. Afterwards, cells were exposed to a 100 μg/ml lipopolysaccharide (LPS) solution for 12 h. The culture medium was separated from cell pellet and was used, without any dilution, to test cytokine production by a Bio-Plex Pro mouse Cytokine Chemokine and Growth Factor Plex Panel kit (Bio-Rad Laboratories, USA) for Interleukin 6 (IL-6) and basic fibroblast growth factor (bFGF). A Bio-Plex machine

(Bio-Plex 200 System) was used. The sensitivity of this method was <10 pg/ml, and assay accuracy is verified in the 1–32,000 pg/ml range.

2.8. Statistical analyses

Quantitative data are expressed as mean value ± standard deviation (SD). Differences among treatment groups were assessed by χ^2 tests for categorical variables or using one-way analysis of variance (ANOVA) followed by Scheffe's post hoc test for continuous variables. p values <0.05 and <0.001 were considered statistically significant and statistically highly significant, respectively.

3. Results and discussion

The production of solid pharmaceutical dosage forms involves several industrial processes, such as sieving, pouring, mixing and kneading. These processes are strongly affected by powder properties, in particular, flowability and bulk density. For example, a cohesive powder is detrimental for the quality of the final product and leads to unsatisfactory product uniformity. More in detail, in the development and manufacturing of granulates, both as oral dosage forms per se, or as intermediates for the preparation of tablets, a suitable flowability is crucial. In particular, wet granulation is useful to this aim and, hence, is very helpful to improve the physical properties of the drugs. In the present study, SMNZ was used as an excipient for the preparation of granules intended for the sustained release of DS. We have previously shown that SMNZ as such possess poor flow properties and, for this reason, we have formulated a granulate to improve flowability features (de Gennaro et al., 2015).

The granulate was obtained by the wet granulation method, according to the indications of the European Pharmacopoeia (8th Ed). The median surface diameter of the granulate was found to be (805 ± 131) μm, with an average (0.750 ± 0.084) circularity value and a (2.16 ± 0.64) aspect ratio. The equivalent slab corresponding to the average granule was determined to have 1444 × 669 μm dimensions.

Granulate flowability was assessed by Carr's index CI(%), Hausner ratio (HR) and angle of repose (α_R). CI (%) is an indicator of powder flowability and, as indicated in Italian Pharmacopoeia, XII ed., a CI(%) < 15 corresponds to a good powder flowability, whereas CI(%) >25 identifies a cohesive powder. The angle of repose, α_R, is related to the interparticle friction. In general, a 31–35° angle indicates good flow properties. The calculated values of CI(%) were 16.4% ± 0.4 and 16.6% ± 0.3 for placebo and DS-loaded granules, respectively, therefore indicating satisfactory flow properties of the granulate, suitable for oral dosage form manufacturing processes. The average HR was 1.28 ± 0.06, and α_R value was 34.1° ± 1.0. The calculated CI (%), HR and α_R point at a “fair/good”, “fair/good” and “passable” flowability, while our previous results for bare SMNZ indicated a “very poor” flowability (United States Pharmacopoeia, 2016). Consequently, melt granulation caused a strong improvement of the flow properties for the tested zeolite. This evident enhancement of granule flowability compared to SMNZ alone can be reasonably ascribed to the increase in particle size due to the granulation itself which, in turn, led to a reduction of interparticle Van der Waals, electrostatic, and capillary forces. Furthermore, the increase in size is associated to gravitational forces, which prevail over interparticle forces, thereby enhancing the flowability of the particulate system (Crowder and Hickey, 2000).

The adsorption isotherm of the granulate is shown in Fig. 1. Here it can be noticed that the sample undergoes a 15% of mass increase thus pointing a limited hygroscopicity of the granulate. More in detail, the mass increase is linear up to 0.75 RH value, while curving upward in the final part of the experiment. This may be due to an initial phase of water absorption hampered by the limited superficial porosity of the sample. Indeed, the measured BET Surface Area is 2.32 m^2/g which is a much lower value compared to the bare zeolite samples (equal to 17.67 m^2/g for CLI_CA). Afterward when the mechanical resistance of the granule is reduced by the water uptake, its intrusion within the granule bulk is favored.

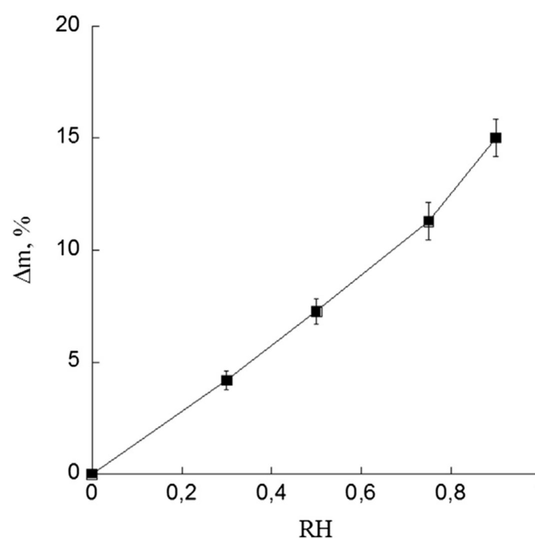

Fig. 1. Moisture sorption curve of the prepared granulate.

The obtained granulate satisfied the uniformity drug content assay which was performed following Pharmacopoeia indications as described above (see Materials and Method section). Indeed, DS content, averaged on ten aliquots of granules after 6 h of granulate dissolution, was found to be 87.6–112.8% of average drug content, therefore pointing out at a uniform mixing of the granulate DS release kinetics from bare SMNZ and granulate were studied. In both cases, a sustained drug delivery was observed. In particular, DS release from bare SMNZ was triggered by ion exchange and mainly driven by diffusion, with a significant burst in the first hour followed by a slower release phase up to 5 h, similarly to our previous results (de Gennaro et al., 2015). In the case of granules, DS release was slower and was completed in approximately 9 h, with approximately 22% of the loaded drug delivered within the first 2 h (i.e. in a time period compatible with the residence time within the gastric environment). Actually, the slowing of the drug delivery rate can be reasonably ascribed to the combination of ion exchange kinetics and the transport hindrances encountered by the drug within the matrix of the granules. Dissolution profiles of the granules are presented in Fig. 2. In particular, the overall DS release was observed to follow quasi-zero order kinetics up to 8 h, irrespective of the pH of the release medium ($r^2 > 0.99$). The DE of the granulate was slightly lower compared to bare SMNZ (56.9 vs. 62.9%), indicating a role of the excipients in slowing down the entire release phenomenon and particularly the initial phase. In particular, it can be noticed that DS delivery is slightly slower at the very initial times, most probably due to the low porosity which slows down water intrusion and subsequent drug dissolution. Thereafter, a quasi-linear release has been found out. These results point at the role of the used excipients in controlling the release rate of the loaded molecule.

Studies of granulate cytotoxicity were carried out in order to compare the effect on the cells of SMNZ, in the free form and also when formulated together with the other excipients of the granulate dosage form. Here, the cytotoxicity elicited by the granulate was evaluated after 24 h of incubation with macrophages and, as shown in Fig. 3A, no significant toxicity was observed for concentrations up to 5 mg/ml. These data indicate the biological safety of the formulation, therefore confirming its possible use in pharmaceutical applications. Moreover, cytotoxicity profiles have shown that DS formulated in the granulate is less cytotoxic than the free drug (Fig. 3B), probably due to the slower release of the drug from the dosage form in the culture medium. In particular, the dose-response curve of the free DS has shown that IC50 was 0.3 mM. Instead, the viability of macrophages was reduced only by 10–

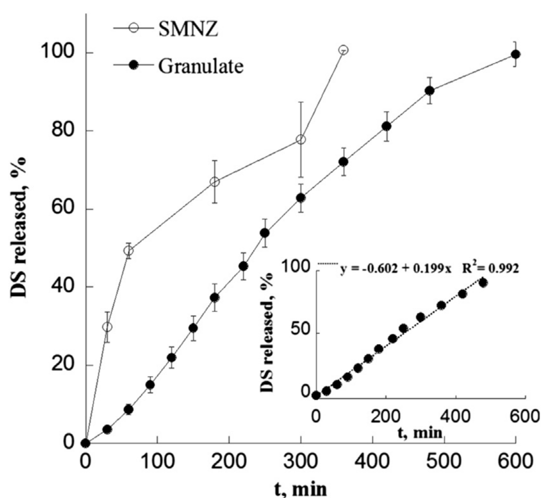

Fig. 2. In vitro DS release profile from of the granulate in simulated gastric fluid (SGF, pH = 1.2) for 2 h followed by dissolution in simulated intestinal fluid (SIF, pH = 6.8) at 37 °C. Inset: DS release profile from the granulate fit to a zero-order equation up to 8 h. Standard deviations have been calculated from at least three independent replicas.

15% ($p < 0.001$) when the cells were incubated in the presence of DS-loaded granulate, at the same concentration of the drug (corresponding to 2 mg/ml of granulate).

Furthermore, in order to investigate the pharmacological activity of the granulate, the cytokine analysis was performed. Actually, DS is a non-steroidal anti-inflammatory drug (NSAID) used in the treatment of a wide range of conditions, such as mild to moderate pain, along with signs and symptoms of inflammatory diseases, such as backache tendonitis, rheumatoid arthritis, osteoarthritis, dislocations, and fractures.

However, to date little is known about the effects of NSAIDs on the production of proangiogenic factors and cytokines, such as bFGF and IL-6, by normal macrophages. Recently, it has been shown that in pro-inflammatory conditions, DS reduces the proliferative effect of LPS according to a dose-dependent pattern. Furthermore, it was also demonstrated that DS is able to inhibit LPS-induced secretion bFGF, to an extent proportional to the dosage (You et al., 2013; Wiktorowska-Owczarek, 2014). This indicates an interesting role of this drug in the regulation of cytokine secretion in normal cells. Furthermore, it is widely recognized that IL-6 is produced at the site of inflammation and plays a major role in the response during the acute phase of inflammation as defined by a variety biological features, such as the production of acute-phase proteins (Gabay, 2006). Also, bFGF is a potent angiogenic factor and endothelial cell mitogen and, interestingly, bFGF levels are increased in chronically inflamed tissue. Finally, it has been also demonstrated that bFGF, bFGF, while not having a direct proinflammatory action, synergistically enhances the mediator-induced recruitment of leukocytes to inflammation, thereon increasing the up-regulation of cell adhesion molecules (CAMs) on endothelium (Zittermann and Issekutz, 2006). On the basis of these findings, we have decided to investigate the anti-inflammatory effects against macrophages exposed to lipopolysaccharide (LPS) of DS, either in its free form or loaded in/on SMNZ, by evaluating bFGF and IL-6 production from 1 h up to 6 h of pre-incubation. First, as shown in Fig. 4, the treatment of macrophages with LPS determined an increased secretion of both cytokines, and this response is related to the presence of TLR4 on the membrane of macrophages as it is well known in the literature (Du et al., 1999). Pretreatment with both free DS or DS-loaded granulate, at the indicated concentrations, significantly decreased the levels of IL-6 and bFGF in macrophages (Fig. 4). In particular, DS pretreatment, at an equivalent DS dose of 0.2 mM, reduced the magnitude of the increase in IL-6 and bFGF cellular levels after 1 h of incubation, approximately by 74.0 and 67.4% respectively, compared to non-pretreated cells (Fig. 4A, B) and the anti-inflammatory effects was reduced with increasing incubation time (1 to 6 h), therefore indicating that the drug is metabolized by the cells in the time frame of the pretreatment. Interestingly, the pretreatment with DS-loaded granulate showed an anti-inflammatory effect that was increasing with increasing incubation time, with a maximum effect after 4 h of incubation, due to the relatively slow drug release; on the contrary, in the case of the treatment with free DS, the entire drug amount was administered to cells at the beginning of the experiment. In detail, the increase in cellular levels of IL-6 and bFGF after cells pre-treatment for 4 h with DS-loaded granulated to an equivalent concentration equal to 0.2 mM have been reduced by approximately 75 and 70%, compared to the cells which did not undergo pre-treatment. (Figs. 4C, D). After 6 h of incubation, the anti-inflammatory effects started to decline, therefore indicating the onset of drug metabolism. Therefore, the results obtained in in vitro cell experiments have shown the ability of the DS-loaded granulate to exert an anti-inflammatory activity on macrophages. In detail, compared to the treatment of cells with free DS, the drug-loaded granulate has shown a different pharmacological profile, with a prolonged anti-inflammatory action in virtue of the granulate ability to sustain DS release.

4. Conclusions

DS-loaded granules containing CLI_CA with suitable technological features have been successfully prepared by the wet granulation method, utilizing HPMC as a binding/controlled release excipient carrier. The utilized method allowed to obtain granules with a satisfactory dosage uniformity and a flowability suitable for a manufacturing process. The produced granules allowed a prolonged DS release compared to the delivery kinetics obtained from bare SMNZ, up to 9 h. The granules did not elicit significant cytotoxicity and could also induce an anti-inflammatory effect on RAW264.7 cells, along with the beginning of drug metabolism for prolonged pretreatment. Taken all together, these data

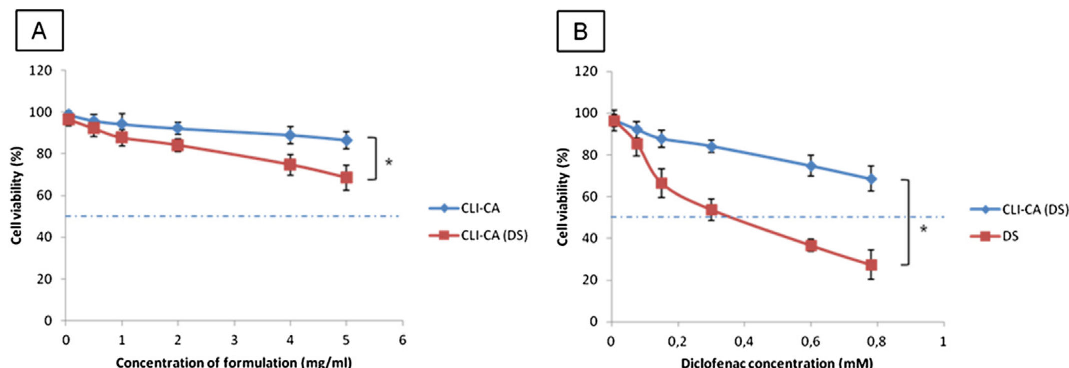

Fig. 3. RAW264.7 cell viability (±SEM) performed by modified MTT method as a function of the granulate concentration (CLI-CA, CLI-CA(DS), mg/ml) (A) and of diclofenac (CLI-CA (DS) and DS, mM) (B). $^{*}p < 0.001$.

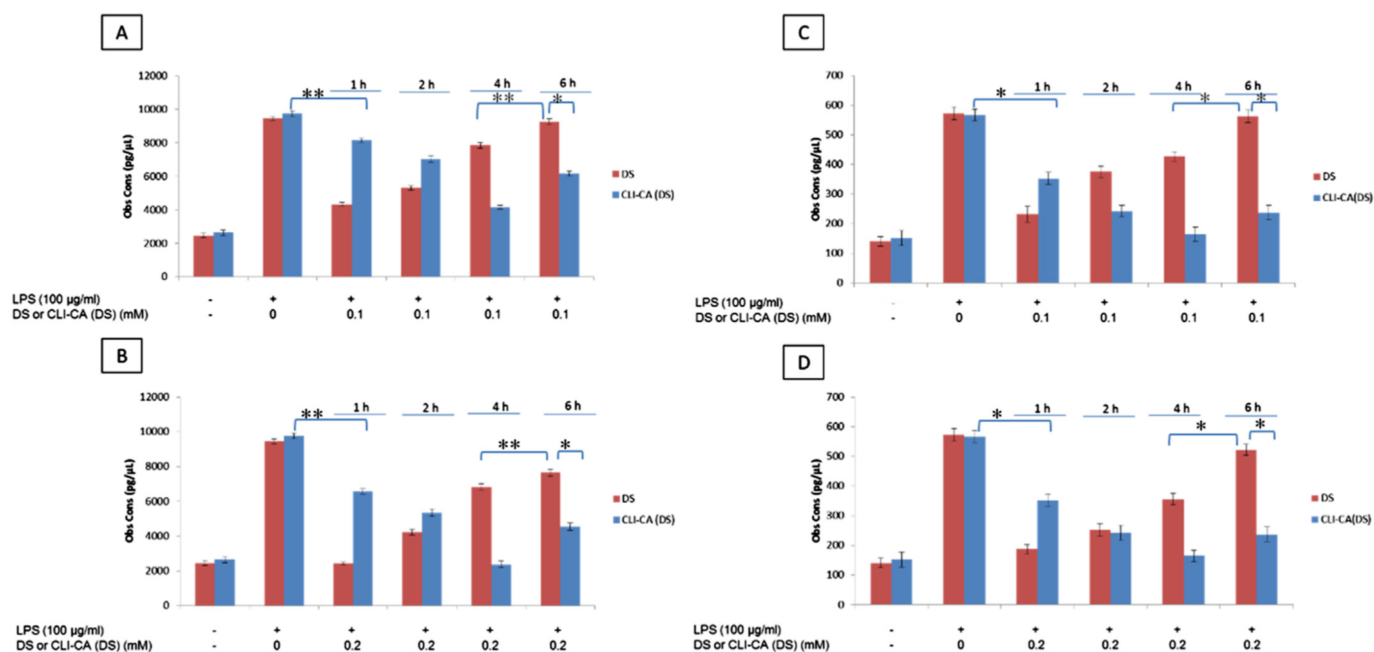

Fig. 4. The effect of free DS and of the granulate on IL-6 (A, B) and bFGF (C, D) productions on RAW264.7 cell line (1.2×10^5 cells/well). Cells were treated with or without 0.1 ml of a 0.1 mM (A, C) and a 0.2 mM solution (B, D) of DS or CLI-CA(DS) for 1, 2, 4 and 6 h before being exposed to LPS (100 μg/ml) for 12 h. $^*p < 0.001$; $^{**}p < 0.05$.

confirm the feasibility of using CLI_CA as an excipient for the production of granules with sustained release features. Further improvements of the flow properties of the granule may obtained through a fluid bed granulation technique, which allows to obtain more rounded structures, thereby reducing the interparticle friction and, hence, the resistance to the flow.

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References

Adamis, Z., Tatrai, E., Honma, K., Six, E., Ungvary, G., 2000. In vitro and in vivo tests for determination of the pathogenicity of quartz, diatomaceous earth, mordenite and clinoptilolite. Ann. Occup. Hyg. 44, 67–74.

Armbruster, T., Gunter, M.E., 2001. Crystal structures of natural zeolites, natural zeolites: occurrence, properties, applications. Rev. Mineral. Geochem. 45, 1–68.

Bonferoni, M.C., Cerri, G., de' Gennaro, M., Juliano, C., Caramella, C., 2007. Zn2+-exchanged clinoptilolite-rich rock as active carrier for antibiotics in anti-acne topical therapy - in-vitro characterization and preliminary formulation studies. Appl. Clay Sci. 36, 95–102.

Cappelletti, P., Colella, A., Langella, A., Mercurio, M., Catalanotti, L., Monetti, V., de Gennaro, B., 2015. Use of surface modified natural zeolite (SMNZ) in pharmaceutical preparations part 1. Mineralogical and technological characterization of some industrial zeolite-rich rocks. Microporous Mesoporous Mater. 224 in press. 10.1016/j.micromeso.2015.05.048.

Carretero, M.I., Pozo, M., 2009. Clay and non-clay minerals in the pharmaceutical industry part I. Excipients and medical applications. Appl. Clay Sci. 46, 73–80.

Carretero, M.I., Pozo, M., 2010. Clay and non-clay minerals in the pharmaceutical and cosmetic industries part II. Active ingredients. Appl. Clay Sci. 47, 171–181.

Cerri, G., de' Gennaro, M., Bonferoni, M.C., Caramella, C., 2004. Zeolites in biomedical application: Zn-exchanged clinoptilolite-rich rock as active carrier for antibiotics in anti-acne topical therapy. Appl. Clay Sci. 27, 141–150.

Conte, C., Scala, A., Siracusano, G., Sortino, G., Pennisi, R., Piperno, A., Miro, A., Ungaro, F., Sciortino, M.T., Quaglia, F., Mazzaglia, A., 2016. Nanoassemblies based on non-ionic amphiphilic cyclodextrin hosting Zn(II)-phthalocyanine and docetaxel: design, physicochemical properties and intracellular effects. Colloids Surf. B: Biointerfaces 146, 590–597.

Crowder, T.M., Hickey, A.J., 2000. The physics of powder flow applied to pharmaceutical solids. Pharm. Technol. 24, 50–58.

Danilczuk, M., Dlugopolska, K., Ruman, T., Pogocki, D., 2008. Molecular sieves in medicine. Mini-Rev. Med. Chem. 8, 1407–1417.

de Gennaro, B., Catalanotti, L., Cappelletti, P., Langella, A., Mercurio, M., Serri, C., Biondi, M., Mayol, L., 2015. Surface modified natural zeolite as a carrier for sustained diclofenac release: a preliminary feasibility study. Colloids Surf., B 130, 101–109.

de Gennaro, B., Mercurio, M., Cappelletti, P., Catalanotti, L., Daković, A., De Bonis, A., Grifa, C., Izzo, F., Kraković, M., Monetti, V., Langella, A., 2016. Use of surface modified natural zeolite (SMNZ) in pharmaceutical preparations. Part 2. A new approach for a fast functionalization of zeolite-rich carriers. Microporous Mesoporous Mater. 235, 42–49.

Du, X., Poltorak, A., Silva, M., Beutler, B., 1999. Analysis of Tlr4-mediated LPS signal transduction in macrophages by mutational modification of the receptor. Blood Cell Mol. Dis. 25, 328–338.

Gabay, C., 2006. Interleukin-6 and chronic inflammation. Arthritis Res. Ther. 8, S3.

Grce, M., Pavelic, K., 2005. Antiviral properties of clinoptilolite. Microporous Mesoporous Mater. 79, 165–169.

Karolewicz, B., 2016. A review of polymers as multifunctional excipients in drug dosage form technology. Saudi Pharm. J. 24, 525–536.

Katic, M., Bosnjak, B., Gall-Troselj, K., Dikic, I., Pavelic, K., 2006. A clinoptilolite effect on cell media and the consequent effects on tumor cells in vitro. Front. Biosci. 11, 1722–1732.

Khan, K.A., 1975. The concept of dissolution efficiency. J. Pharm. Pharmacol. 27, 48–49.

Li, Z.H., Bowman, R.S., 1998. Sorption of perchloroethylene by surfactant-modified zeolite as controlled by surfactant loading. Environ. Sci. Technol. 32, 2278–2282.

Mayol, L., Serri, C., Menale, C., Crispi, S., Piccolo, M.T., Mita, L., Giarra, S., Forte, M., Saija, A., Biondi, M., Mita, D.G., 2015. Curcumin loaded PLGA-poloxamer blend nanoparticles induce cell cycle arrest in mesothelioma cells. Eur. J. Pharm. Biopharm. 93, 37–45.

Miro, A., d'Angelo, I., Nappi, A., La Manna, P., Biondi, M., Mayol, L., Musto, P., Russo, R., La Rotonda, M.I., Ungaro, F., Quaglia, F., 2013. Engineering poly(ethylene oxide) buccal films with cyclodextrin: a novel role for an old excipient? Int. J. Pharm. 452, 283–291.

Moyano-Mendez, J.R., Fabbrocini, G., De Stefano, D., Mazzella, C., Mayol, L., Scognamiglio, I., Carnuccio, R., Ayala, F., La Rotonda, M.I., De Rosa, G., 2014. Enhanced antioxidant effect of trans-resveratrol: potential of binary systems with polyethylene glycol and cyclodextrin. Drug Dev. Ind. Pharm. 40, 1300–1307.

Mumpton, F.A., 1999. La roca magica: uses of natural zeolites in agriculture and industry. Proc. Natl. Acad. Sci. U. S. A. 96, 3463–3470.

Pavelic, K., Hadzija, M., Bedrica, L., Pavelic, J., Dikic, I., Katic, M., Kralj, M., Bosnar, M.H., Kapitanovic, S., Poljak-Blazi, M., Krizanac, S., Stojkovic, R., Jurin, M., Subotic, B., Colic, M., 2001. Natural zeolite clinoptilolite: new adjuvant in anticancer therapy. J. Mol. Med. 78, 708–720.

Rivera, A., Farias, T., 2005. Clinoptilolite-surfactant composites as drug support: a new potential application. Microporous Mesoporous Mater. 80, 337–346.

Rodriguez-Fuentes, G., Barrios, M.A., Iraizoz, A., Perdomo, I., Cedre, B., 1997. Enterex: anti-diarrheic drug based on purified natural clinoptilolite. Zeolites 19, 441–448.

Rodriguez-Fuentes, G., Denis, A.R., Alvarez, M.A.B., Colarte, A.I., 2006. Antacid drug based on purified natural clinoptilolite. Microporous Mesoporous Mater. 94, 200–207.

Serri, C., de Gennaro, B., Catalanotti, L., Cappelletti, P., Langella, A., Mercurio, M., Mayol, L., Biondi, M., 2016. Surfactant-modified phillipsite and chabazite as novel excipients for pharmaceutical applications? Microporous Mesoporous Mater. 224, 143–148.

Shen, S.C., Ng, W.K., Chia, L.S., Dong, Y.C., Tan, R.B., 2013. Applications of mesoporous materials as excipients for innovative drug delivery and formulation. Curr. Pharm. Des. 19, 270–289.

United States Pharmacopeia 39, 2016. The United State Pharmacopeial Convention Twinbrook Parkway, Rockville.

Viseras, C., Aguzzi, C., Cerezo, P., Lopez-Galindo, A., 2007. Uses of clay minerals in semisolid health care and therapeutic products. Appl. Clay Sci. 36, 37–50.

Wiktorowska-Owczarek, A., 2014. The effect of diclofenac on proliferation and production of growth factors by endothelial cells (HMEC-1) under hypoxia and inflammatory conditions. Acta Pharma. 64, 131–138.

Yang, P., Gai, S., Lin, J., 2012. Functionalized mesoporous silica materials for controlled drug delivery. Chem. Soc. Rev. 41, 3679–3698.

You, S., Nakanishi, E., Kuwata, H., Chen, J., Nakasone, Y., He, X., He, J., Liu, X., Zhang, S., Zhang, B., Hou, D.X., 2013. Inflammatory effects and molecular mechanisms of garlic organosulfur compounds on the production of inflammatory mediators. Mol. Nutr. Food Res. 57, 2049–2060.

Zittermann, S.I., Issekutz, A.C., 2006. Basic fibroblast growth factor (bFGF, FGF-2) potentiates leukocyte recruitment to inflammation by enhancing endothelial adhesion molecule expression. Am. J. Pathol. 168, 835–846.

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