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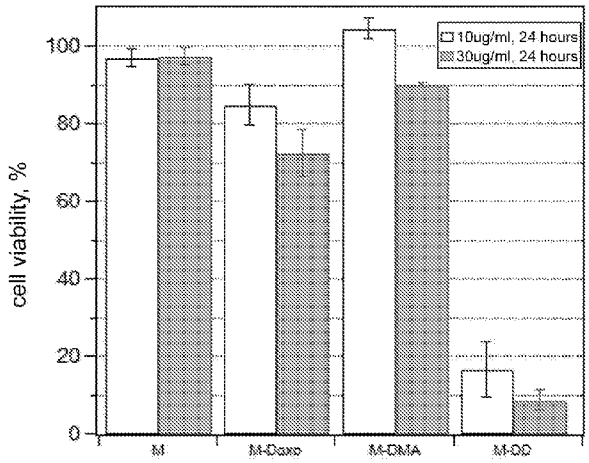

FIG. 2

(57) Abstract: A nanocarrier is described loaded with at least one inhibitor of exocytosis and at least one active ingredient. Said nanocarrier is used for medical use, in particular in the treatment of a tumour.

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NANOCARRIER COLOADED WITH AN INHIBITOR OF EXOCYTOSIS AND AN ACTIVE INGREDIENT

TECHNICAL FIELD

The present invention concerns a nanocarrier loaded with at least one inhibitor of exocytosis and at least one active ingredient.

Micro and nanostructured particles are studied as carriers for the transport of drugs in order to selectively direct the latter to the target tissue, increasing the effectiveness and minimizing the side effects thereof. The further general advantages of this type of carrier are: the possibility of administering molecules that are non-soluble or have a low solubility in water and protection against premature degradation of the drug. For these reasons, the use of certain drugs coupled with nanocarriers is already in the clinical study phase.

STATE OF THE PRIOR ART

In recent years increasing interest has been shown in the use of mesoporous silica nanoparticles (MSN). In fact, silicon oxide has been recognised as a biocompatible safe material by the Food and Drug Administration (FDA). Furthermore, due to their considerable versatility and high drug loading capacity, MSN are an excellent means of carrying drugs in an effective safe manner.

In the oncological field, effective safe delivery of drugs is particularly important. Doxorubicin, an anthracycline administered for the treatment of various types of tumour including tumours of the blood, solid tumours and sarcomas, is one of the most widely used drugs.

In general, once the nanoparticles loaded with the drug have reached the target cells, they are internalized by endocytosis. The process of endocytosis can be classified according to the membrane proteins involved which in turn depend on the

chemical/physical characteristics of the carrier being studied. Generally speaking, the process entails 4 phases:
- interaction with the cell membrane;
- invagination of the membrane;
- formation of vesicles (primary and secondary endosomes);
- fusion of the vesicles with a subcellular compartment and release of the material transported into the cell.

In particular, the MSN, once internalized, are directed towards the endo-lysosomal pathway, in which the lysosomes represent the last cellular compartment reached. The endo-lysosomal pathway is characterized by a gradual reduction in the pH which decreases from 7.4 in the plasmatic membrane to approximately 6 in the primary endosomes, 5.5 in the secondary endosomes and lastly to 4.6-5 in the lysosomes.

In general, the principle underlying the administration of drugs by means of nanoparticles is the so-called "Trojan horse effect", i.e. the effect by which the nanoparticles are internalized in cells rapidly and in very large quantities, contrary to what happens with loaded molecules (drugs, oligonucleotides, etc.), which do not cross the cell membrane as easily.

To further increase the therapeutic effect of the nanocarriers, new strategies are being sought which can locally increase the drug levels. For example, it has been recently reported that an appropriate chemical modification of the surface of the nanoparticle can induce the so-called "endolysosomal escape" in order to obtain release of the active molecules directly into the cytosol, maximizing the specific action of the drug transported. Other strategies aim at functionalization with specific molecules, for example by means of "cell penetrating peptides", in order to favour cell uptake of the nanocarriers, thus increasing the final drug dose.

Some studies have focused on understanding of the mechanisms underlying the processes leading to endocytosis of nanoparticles and how the chemical-physical characteristics of the nanomaterial can influence the particle/cell interaction.

The natural processes of cell exocytosis pose a general limit to the nanocarriers; according to these processes, as the nanoparticles are abundantly internalized by the cells, the nanocarriers internalized are just as effectively expelled to the outside of the cells. These processes significantly reduce the mean intracellular "residence" time of the nanocarriers, thus considerably reducing the therapeutic effectiveness. So far, these problems have not been extensively investigated because little is still known about the molecular and cellular mechanisms that regulate the exocytosis of nanoparticles and nanocarriers.

A few recent articles consider the phenomenon of exocytosis of nanoparticles studying the correlation with the chemical-physical properties of the carriers analysed, but without succeeding in providing a detailed description of the mechanisms underlying these events. One of the hypotheses concerning the mechanism of exocytosis of nanoparticles is described in Yanes et al. (Small 9, 697-704, 2013) and supposes that the exocytosis occurs directly via fusion of the lysosomes with the cell membrane, the so-called lysosomal exocytosis, but the data available in the literature are still insufficient to clarify the phenomenon.

Characterization of the mechanisms that determine the expulsion of nanocarriers from the cell is essential to better understand the real potential of the use of nanomaterials in a drug delivery system. In fact ideally it is fundamental to retain the nanocarriers inside the cell for a sufficient length of time for them to carry out their functions optimally. The greatest limitation of this type of approach is the lack of selectivity

of the molecules normally used to inhibit the process of exocytosis. It is known, in fact, that many mechanisms that govern exocytosis are the same as those that regulate the processes of endocytosis and that the inhibitors used so far to study the exocytosis of nanocarriers interfere with the process of endocytosis. For this reason these inhibitors, in vitro, are always administered in a phase subsequent to the phase of administration of the nanocarrier bearing the drug, so that the initial uptake of the drug by the cells is not inhibited. In vivo this sequential and separate administration would be toxic for the organism and therefore not applicable.

So far, therefore, no nanocarriers are available that have an action targeted at inhibiting the release of the carriers from the cells.

SUBJECT OF THE INVENTION

One object of the present invention is therefore to develop nanocarriers which can be loaded with drugs, which are effectively internalized in the cells and are not expelled to the outside of the cells, and which are safe and without side effects in particular for in vivo administration.

The above-mentioned object is achieved by the present invention since it concerns a nanocarrier as defined in claim 1.

Definitions

The term “nanocarrier” indicates a material formed of particles with dimensions in the order of 1 to 500 nm able to transport another substance, for example a drug.

The expression “nanocarrier loaded with the substance X” indicates a nanocarrier to which the substance X is bonded, or in which the substance X is encapsulated/trapped, or with which the substance X is associated.

The expression “nanocarrier coloaded with substance X and

substance Y" or "nanocarrier coloaded with a combination of substance X and substance Y" indicates a nanocarrier to which substance X and substance Y are bonded, or in which substance X and substance Y are encapsulated/trapped, or with which substance X and substance Y are associated.

Description of the Figures

- figure 1 shows a graph with ICP-AES measurements (atomic emission spectroscopy), in which the silicon content per cell (mol/cell) is evaluated after exposure to nanoparticles for different times;
- figure 2 contains a graph which shows the cell viability measured via WST assay after treatment with 10 µg/ml and 30 µg/ml of non-functionalized nanoparticles (M), loaded with doxorubicin (M-doxo), DMA (M-DMA), or coincubated with doxorubicin and DMA (M-DD) for 24 hours;
- figure 3 contains a graph which shows the cell viability measured via WST assay after treatment with 30 µg/ml of non-functionalized nanoparticles (M), loaded with doxorubicin (M-Doxo), cytochalasin B (M-CytB), or coincubated with doxorubicin and cytochalasin B (M-DC) for 24 hours;
- figure 4 contains a graph which shows the release of doxorubicin in solution by MSN coincubated with doxorubicin and DMA at pH 4.5 and 7.4.

Detailed disclosure

The nanocarrier according to the present invention is coloaded with at least one inhibitor of exocytosis and at least one active ingredient.

The nanocarrier is preferably a metal nanocarrier (nanoparticles of gold, silver, platinum, iron oxide, cerium oxide, silicon oxide, mesoporous silicon/silica, zinc oxide, quantum dots (CdSe, InP), titanium oxide, copper), a polymer nanocarrier (particles of PLGA; PLA, chitosan, caprolactones, layer-by-layer capsules, dendrimers), a lipid nanocarrier

(liposomes, lipoplexes, "solid lipid" particles), a carbon nanocarrier (nanodiamonds, nanoparticles of graphene/graphene oxide, nano-onions of carbon, carbon dots, nanotubes of carbon, fullerenes) or a protein and oligonucleotide nanocarrier.

More preferably the nanocarrier is a mesoporous silica nanoparticle.

The exocytosis inhibitor is preferably dimethyl amiloride (5-(N,N-dimethyl)amiloride hydrochloride), cytochalasin A, B or D, or nocodazole. The cytochalasin A and the cytochalasin D act by inhibiting polymerization of the actin. The nocodazole is an inhibitor of the formation of microtubules. More preferably the exocytosis inhibitor is dimethyl amiloride or cytochalasin B. Even more preferably, the inhibitor is dimethyl amiloride.

The active ingredient is preferably a drug, a plasmid or a miRNA/siRNA, more preferably an antitumor drug, even more preferably doxorubicin.

The nanocarrier according to the present invention is used for the treatment of a medical pathology. More in particular the above-mentioned nanocarrier is used for the treatment of a tumour.

Examples

Example 1 – exocytosis of nanoparticles

Figure 1 illustrates an example of how the nanoparticles are normally expelled outside the cell by means of an efficient process of exocytosis.

The cellular internalization over time of nanoparticles of silicon oxide functionalized on the surface with amino groups (NH_2) (50 nm diameter, produced by HiQ-Nano), widely used in nanomedicine due to their biocompatibility and versatility, was analysed.

A solution of said nanoparticles at a concentration of 1 nM was added to HeLa cell cultures. ICP-AES measurements (inductively coupled plasma atomic emission spectroscopy) were taken to evaluate the silicon content per cell (mol/cell) after exposure to the nanoparticles for different times.

It can be clearly seen that, vis-à-vis a rapid and massive cell internalization of nanoparticles, there is an equally efficient process of exocytosis which causes a significant expulsion of the nanoparticles from the cell.

If said nanoparticles were used by nanocarriers transporting drugs, therefore, the phenomenon of exocytosis would substantially reduce their therapeutic effectiveness, regardless of the active drug/molecule transported.

Example 2 – Mesoporous silica nanoparticles loaded with doxorubicin and dimethyl amiloride

A solution of mesoporous silica nanoparticles (MSN) produced by Sigma Aldrich (CAS number: 7631-86-9) at a concentration of 1 mg/ml was prepared by adding 0.5 mg/ml of doxorubicin and 0.5 mg/ml of dimethyl amiloride (DMA) and left under stirring for one night to favour the diffusion of both the molecules inside the MSN pores. The mean value of the diameter of the MSN is 200 nm and the porosity 4 nm.

The MSN were first centrifuged and thoroughly washed and the quantities of doxorubicin and DMA absorbed were determined by difference, reading the absorbance of the supernatant at 495 nm and 375 nm respectively and quantifying by means of calibration curve.
The MSN prepared in this way were then resuspended in culture medium at a final concentration of 10 and 30 µg/ml and placed in contact with HeLa cells for different times.

A cell viability assay (WST) was used to test the HeLa cells treated with different concentrations of MSN coincubated with doxorubicin and DMA (M-DD).

Figure 2 contains a summary graph which shows the cell viability after treatment with 10 µg/ml and 30 µg/ml of non-functionalized MSN (M), loaded with doxorubicin (M-doxo), DMA (M-DMA), or coincubated with doxorubicin and DMA (M-DD) for 24 hours.

The results show a clear reduction in metabolically active cells following the treatment with M-DD compared to the treatment with nanoparticles containing exclusively doxorubicin (M-Doxo) (after 24 hours, there is an almost total mortality in the case of the co-incubated system).

The effect observed is due to the lower level of expulsion of the MSN from the cell (exocytosis) by the cell during the moments subsequent to internalization. In general, the nanocarrier according to the invention, by lowering the phenomena of cellular exocytosis of MSN, significantly increases the residence time of the carrier loaded with the drug and consequently the therapeutic effectiveness.

It is very important to note that the methodology proposed offers a strong synergic effect in terms of therapeutic effectiveness. In fact, it can be seen from the data reported in Figure 2 that the therapeutic effectiveness of the co-incubated nanocarrier (induction of almost total cell death) is much greater than the effectiveness that could be expected only with the higher intracellular drug dose deriving from inhibition of the exocytosis. In the case of the doxorubicin alone, it can be seen that although the dose is tripled (from 10 to 30 □g/ml), only a small gain of 10% is obtained in terms of mortality, therefore the coincubated nanocarrier system is able to induce complex synergic effects at cellular level which considerably increase the effectiveness of the drug transported.

Example 3 – Mesoporous silica nanoparticles loaded with doxorubicin and cytochalasin B

Alternatively to the DMA, cytochalasin B was used as an inhibitor of exocytosis.

Figure 3 contains a summary graph which shows the cell viability after treatment with 30 µg/ml of non-functionalized nanoparticles (M), loaded with doxorubicin (M-Doxo), cytochalasin B (M-CytB) and coincubated with doxorubicin and cytochalasin B (M-DC) for 24 hours.

The data obtained from WST test show that also in the presence of the inhibitor an improvement can be observed in the cytotoxic activity of the doxorubicin, even though the inhibitory effect of the Cytochalasin B proved less effective than what was seen with the DMA.

Example 4 – Influence of the pH on release of the drug inside the cell

With reference to Figure 4, it was observed that the release of doxorubicin from the MSN coincubated with Doxo-DMA is strongly influenced by the pH of the solution with a considerable increase in the quantity of drug released with the acidification.

By retaining the nanoparticles for longer inside the lysosomal environment which has an acid pH, it is possible to obtain an increase in the local dose of doxorubicin.

From the above examples, the advantages provided by the nanocarrier according to the present invention are evident.

In particular, the above-mentioned nanocarrier allows the use of lower drug doses, since internalization of the nanocarrier is particularly efficient over time and release of the drug inside the cell is favoured in particular by the acid pH of the

lysosomal environment. The release of the drug is therefore high and prolonged over time.

Furthermore, the processes of lysosomal exocytosis of the internalized nanocarriers are simultaneously inhibited due to the presence of the inhibitor of exocytosis. The inhibitor of exocytosis has no effect on the initial endocytosis of the nanocarrier since it is encapsulated in the latter. Therefore, it does not negatively influence the entry of the nanocarrier into the cell, because it is masked by the nanocarrier itself.

Lastly, as also discussed previously, the nanocarrier coincubated with the drug and the inhibitor of exocytosis induces complex synergic effects at cellular level, hence the therapeutic effectiveness of the drug is strongly and further increased (further with respect to the sole increase of intracellular dose due to inhibition of the exocytosis).

CLAIMS

1. A nanocarrier coloaded with at least one inhibitor of exocytosis and at least one active ingredient.

2. A nanocarrier coloaded with a combination of at least one inhibitor of exocytosis and at least one active ingredient.

3. The nanocarrier according to claim 1 or 2, wherein the nanocarrier is selected from the group consisting of a metal nanocarrier, a polymer nanocarrier, a lipid nanocarrier, a carbon nanocarrier, and a protein and oligonucleotide nanocarrier.

4. The nanocarrier according to claim 3, wherein the nanocarrier is a mesoporous silica nanoparticle.

5. The nanocarrier according to any of the preceding claims, wherein the at least one inhibitor of exocytosis is dimethyl amiloride, cytochalasin A, B or D, or nocodazole.

6. The nanocarrier according to claim 5, wherein the at least one inhibitor of exocytosis is dimethyl amiloride or cytochalasin B.

7. The nanocarrier according to claim 6, wherein the at least one inhibitor of exocytosis is dimethyl amiloride.

8. The nanocarrier according to any of the preceding claims, wherein the at least one active ingredient is a drug, a plasmid or a miRNA or an siRNA.

9. The nanocarrier according to claim 8, wherein the drug is doxorubicin.

10. The nanocarrier according to any of the preceding claims for medical use.

11. The nanocarrier according to any of claims 1 to 10 for use in the treatment of a tumour.

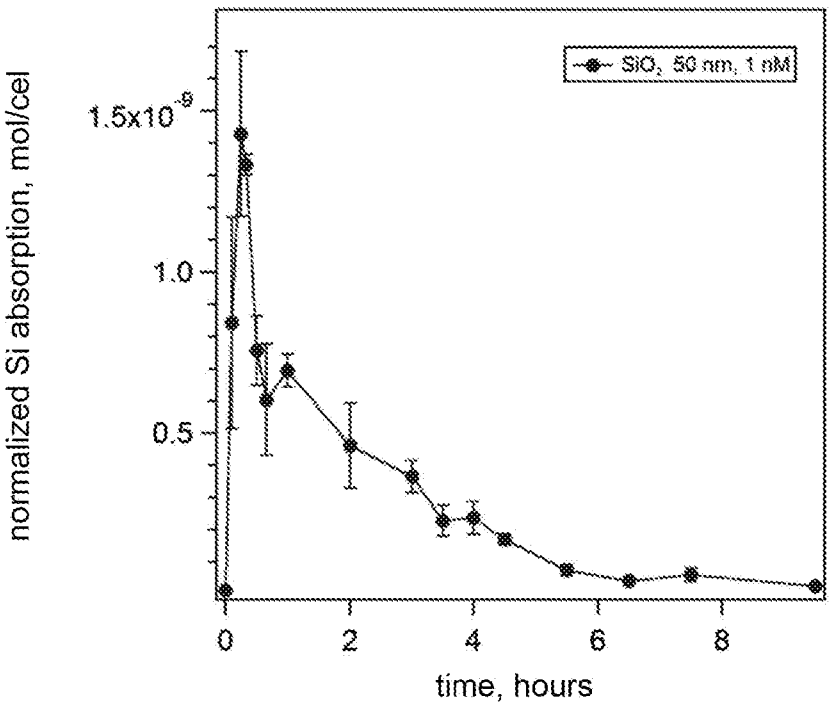
SiO2, 50 nm, 1 nM
normalized Si absorption, mol/cel
1.5x10-9
1.0
0.5
0
2
4
6
8
time, hours

FIG. 1

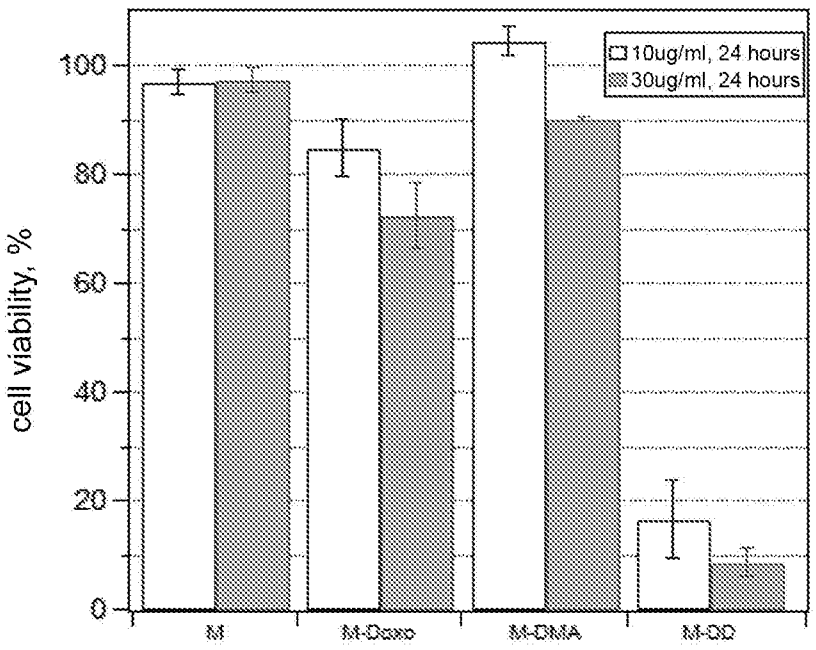
10ug/ml, 24 hours
30ug/ml, 24 hours
cell viability, %
100
80
60
40
20
0
M
M-Doxo
M-DMA
M-OD

FIG. 2

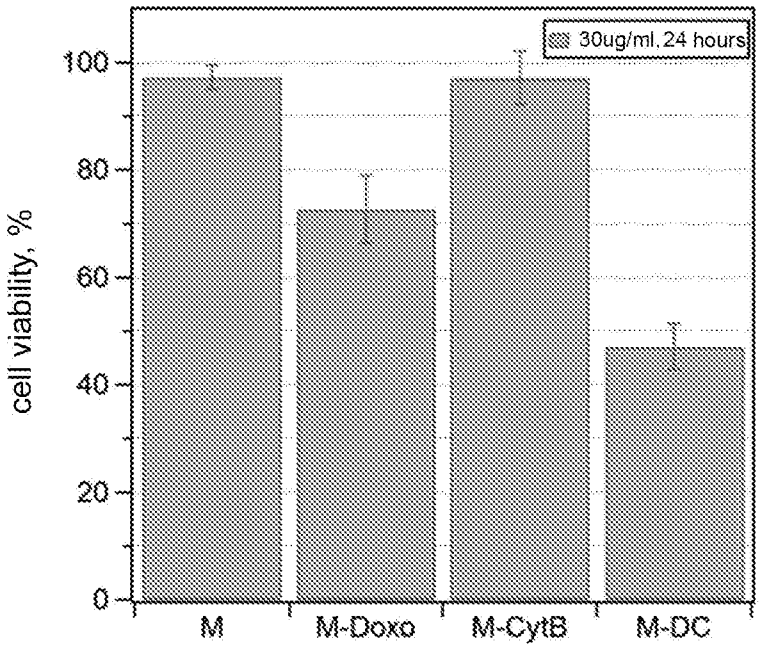
30ug/ml, 24 hours
cell viability, %
100
80
60
40
20
0
M
M-Doxo
M-CytB
M-DC

FIG. 3

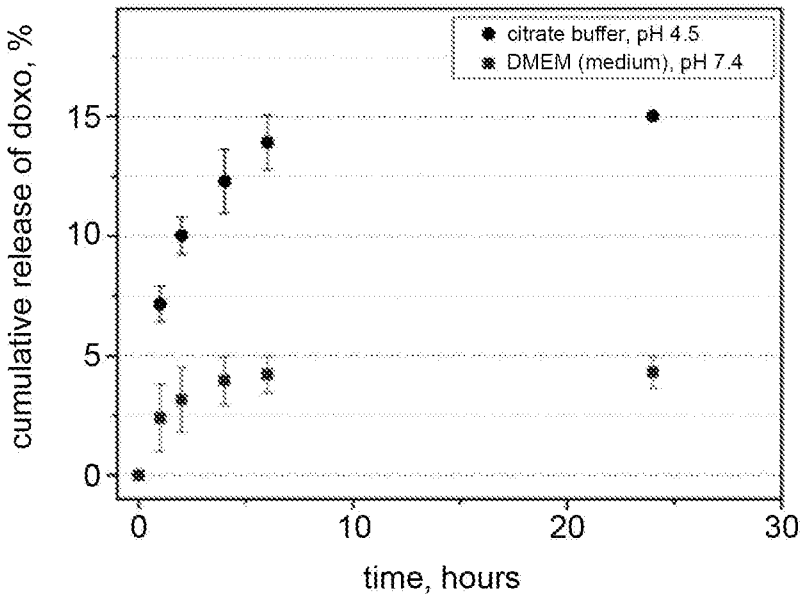
citrate buffer, pH 4.5
DMEM (medium), pH 7.4
cumulative release of doxo, %
15
10
5
0
0
10
20
30
time, hours

FIG. 4

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/050754

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K9/51 A61K9/00
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 663 881 B2 (KUNZ LAWRENCE L [US] ET AL) 16 December 2003 (2003-12-16) column 4, line 46 - line 63 column 21, line 30 - line 60 claims ----- -/--	1-11

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 May 2017	15/05/2017

Name and mailing address of the ISA/	Authorized officer
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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2017/050754

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LÜTFI GENÇ: "Preparation and characterization of nocodazole-loaded solid lipid nanoparticles", PHARMACEUTICAL DEVELOPMENT AND TECHNOLOGY, vol. 19, no. 6, 10 September 2014 (2014-09-10), pages 671-676, XP055309542, US ISSN: 1083-7450, DOI: 10.3109/10837450.2013.819017 abstract page 671, column 2, line 8 - page 672, column 1, line 4 page 672; table 1 page 676, column 1, line 1 - line 11	1-11
A	ROLANDO E. YANES, DERRICK TARN, NGELA A. HWANG, DANIEL P. FERRIS: "Involvement of lysosomal exocytosis in the excretion of mesoporous silica nanoparticles and enhancement of the drug delivery effect by exocytosis inhibition", SMALL, vol. 9, no. 5, 1 January 2013 (2013-01-01) , pages 697-704, XP002763338, cited in the application the whole document	1-11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2017/050754

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6663881 B2	16-12-2003	NONE	