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**Hyaluronated mesoporous silica nanoparticles for active targeting: influence of conjugation method and hyaluronic acid molecular weight on the nanovector properties**

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(Article begins on next page)

1 **Hyaluronated mesoporous silica nanoparticles for active targeting: influence of**  
2 **conjugation method and hyaluronic acid molecular weight on the nanovector**  
3 **properties**

4  
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1 **Abstract**

2 We have prepared and evaluated ~~about their~~ the physico-chemical and biological properties of four  
3 different hyaluronated mesoporous silica nanoparticles (MSNs) samples (MSN/HA). Hyaluronic  
4 acid (HA) with two different molecular weights (200 and 6.4 kDa) was used for the conjugation of  
5 aminopropyl-functionalized MSN (NH<sub>2</sub>-MSN), following two different procedures. Namely,  
6 samples HA200A and HA6.4A were prepared by reacting activated HA with NH<sub>2</sub>-MSN (method  
7 A), while samples HA200B and HA6.4B were obtained carrying out HA activation in the presence  
8 of the nanoparticles (method B). The four samples showed similar hydrophilicity, but clear  
9 differences in the HA loading, textural properties, surface charge and stability of the suspensions.  
10 More in detail, conjugation using low molecular weight HA with method A resulted in low HA  
11 loading, with consequent scarce effects on dispersity and stability in physiological media. The  
12 highest yield and corresponding best performances were obtained with method B using high  
13 molecular weight HA. HA loading and molecular weight also influenced in a concerted way the  
14 biological response towards the MSNs of CD44 target cancer cells (CD44+) and control cells  
15 (CD44-): MDA-MB-231 and A2780, respectively. The absence of cytotoxicity was assessed.  
16 Moreover, the targeting ability of the best performing MSN/HA was confirmed by cellular uptake  
17 studies.

18

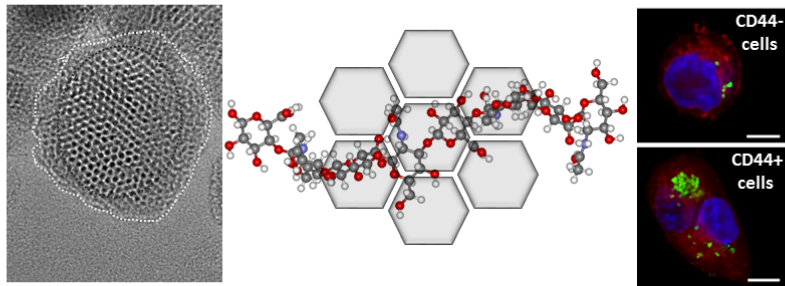
19 **Key words**

20 Hyaluronic acid, mesoporous silica nanoparticles, CD44 receptor, active targeting, hydrophilicity,  
21 infrared, water adsorption, MSN

22

1 **Graphical abstract**

2



3

4 Hyaluronated mesoporous silica nanoparticles (MSN/HA) are preferentially internalized in CD44+

5

tumor cells

6

## 1. Introduction

Nanoparticles-based targeted therapy has emerged in recent years as an innovative strategy to maintain a drug therapeutic dose at the target site, while reducing systemic drug toxicity and adverse side effects to healthy tissues [1-3]. This approach is particularly important in relation to cancer therapy, where the differences in biochemistry between cancerous and normal tissues can be exploited for the selective targeting of over-expressed tumour specific receptors [4-7]. To this aim, nanomaterials are an ideal playground, thanks to their intrinsic properties such as high surface area, tuneable size and shape coupled to ease of synthesis and functionalization. Nanoparticles may be loaded with a plethora of bioactive molecules (*e.g.*, small molecules, peptides, nucleic acids, etc.) to protect them from cleavage by external agents, thus the encapsulated drugs do not participate in the control over pharmacokinetic and biodistribution. Moreover, nanosize ~~allows for~~ **permits** passive transport in biological fluids and for establishing molecular interactions at the cellular and subcellular level [1].

Liposomes [8-10] and biodegradable polymeric nanoparticles [11-13] are among the most versatile biocompatible systems to encapsulate active ingredients. Mesoporous silica nanoparticles (MSNs) can be considered as their inorganic counterparts, with intrinsic features such as a huge available inner volume, inertness and chemical stability [14-17]. The ease of surface functionalization makes them ideal materials to develop “pharmaceutically adapted platforms” [18, 19], with great potentiality in relation to **stimuli responsive** applications [20-26]. One of the major drawbacks for the systemic application of these nanosystems is their poor dispersity in biological fluids, which can be however improved by appropriate surface functionalization [19]. Functionalization **(often carried out to optimize the interaction with the guest drug molecules)** can be obtained with a variety of organosilanes, ~~to optimize the interaction with the guest drug molecules~~ [27]. This has important consequences on the surface properties, such as charge and hydrophilicity [28], which ~~in turns~~ can profoundly ~~affect~~ **influence** the nanoparticle cytotoxicity [29], cellular uptake, transport and/or fate **in biological**

1 fluids of the nanoparticles [30, 31].

2 Surface modification of nanocarriers through macromolecules is particularly relevant in the  
3 field of cancer treatment [32], where the conjugation of cytotoxic drugs with macromolecules is  
4 designed to improve their pharmacokinetic profile, prolonging the distribution and elimination  
5 phases [33]. The most employed are N-(2-hydroxypropyl) methacrylamide (HPMA),  
6 polyglutamate, human serum albumin, dextrans, heparin, chitosan, dendrimers, multi-arm  
7 polyethylene glycol (PEG), and hyaluronic acid (HA) [32, 33]. HA is a naturally-occurring  
8 glycosaminoglycan and a major component of the extracellular matrix. The HA receptor CD44  
9 is overexpressed in many cancer cells, and in particular in tumor-initiating cells. HA has thus  
10 attracted considerable interest in for the development of nanoplatfoms for actively targeting  
11 drugs, genes, and diagnostic agents [34, 35].

12 In recent years, HA-conjugated MSN systems have been proposed in the literature, with the double  
13 effect aim to improve the MSNs dispersity and obtain a targeted delivery to CD44 overexpressing  
14 cancer cells [36-38]. Zhang *et al.* recently proposed biotin-modified HA coupled to MSN to enable  
15 controlled drug release at cancer cells expressing CD44 HA receptor CD44 [39]. Moreover, Chen *et*  
16 *al.* exploited used HA as both capping and targeting agent. In their work, the entrapped guest  
17 molecules were released from the inner pores of MSNs upon HA degradation in response to  
18 hyaluronidase-1, which occurred after receptor-mediated endocytosis into targeted cancer cells [40].

19 The potentiality of HA-conjugated MSN systems was further investigated by developing dual-  
20 stimuli responsive systems. To this aim, the polysaccharide was conjugated to MSNs through a  
21 disulfide bond, which was cleaved in the presence of the high glutathione concentration  
22 characterizing cancer cells [23, 24]. Additionally, multifunctional “theranostic” materials were  
23 developed, coupling the above-mentioned properties to those of a gadolinium based bovine serum  
24 albumin complex for a concomitant simultaneous redox-responsive targeted drug delivery and  
25 magnetic resonance imaging [41].

1 ~~This work reports about~~ **In this work,** ~~the synthesis and characterization of~~ hyaluronated MSNs  
2 (MSN/HA), ~~which~~ have been developed with the double aim to improve their dispersion in  
3 physiological media and their targeting ability. ~~Here~~ **F**or the first time we compare different  
4 synthetic approaches, and the use of HA of different molecular weights (6.4 kDa and 200kDa),  
5 identify the optimal strategy to obtain the better performances, both in terms of biological response  
6 and potential pharmaceutical application. A full physico-chemical description of the produced  
7 hybrid materials is given, including routine characterization, hydrophilicity ~~analysis~~ **assessment,**  
8 molecular and quantitative analysis of the HA external shell. Finally, the biological effects in  
9 cultured cells were studied and the results correlated to the materials properties.

10

## 11 **2. Experimental section**

### 12 *2.1 Materials*

13 Cetyltrimethylammonium bromide (CTAB), tetraethyl orthosilicate (TEOS), sodium hydroxide  
14 (NaOH), (3-aminopropyl)-triethoxysilane (APTS) and all the other reagents and solvents were  
15 purchased from Sigma-Aldrich (Milan, Italy) and employed as received. Sodium hyaluronate (HA,  
16 of molecular weights MW 6.4 and 200 kDa) was purchased from Lifecore Biomedical (Chaska,  
17 MN). Fluorescein-5-isothiocyanate (FITC) was provided by Invitrogen (Life Technologies, Monza,  
18 Italy). MilliQ<sup>®</sup> water was used in all synthetic steps.

19

### 20 *2.2 NH<sub>2</sub>-MSN synthesis*

21 MSN samples were prepared following a slightly modified literature procedure [42, 43]. CTAB (1  
22 g, 2.74 mmol); employed as Structure Directing Agent (SDA),  
23 under stirring and heating. At the stable temperature of 80°C, NaOH (2.0 M, 3.5 ml) was slowly  
24 added to the mixture. TEOS (5 ml, 22.4 mmol) was then added ~~dropwise~~ **drop-wise** ~~over 10 min~~  
25 under vigorous stirring. After 2 h ~~of stirring at 80°C~~ the milky reaction mixture was cooled to room  
26 temperature (RT) and the white precipitate was filtered off and washed with abundant water and

1 methanol. The SDA was removed from the as-synthesized material by calcination at 550 °C,  
2 heating to the desired temperature under N<sub>2</sub> flow and switching to O<sub>2</sub> for a 6 h isotherm.

3 **Aminopropyl-functionalized MSN (NH<sub>2</sub>-MSN)** sample was prepared with APTS by post-synthesis  
4 grafting with a procedure modified from the literature [44, 45]. Namely, 1 g of MSN (overnight  
5 dried at 100°C) were suspended in 30 ml of anhydrous toluene. The particles suspension was heated  
6 at 130°C under stirring. Next, 0.6 ml of APTS were added drop-wise and the mixture was allowed  
7 to reflux for 17 h. The modified NH<sub>2</sub>-MSNs were filtered off and washed with toluene, ethanol,  
8 water and ~~at last~~ **finally** methanol. Subsequently, the sample was dried at 110°C for 3 h ~~to favor the~~  
9 **for curing,** and at 80°C overnight [46].

10

### 11 *2.3 Hyaluronic acid conjugation: MSN/HA samples*

12 Conjugation with HA was carried out starting from NH<sub>2</sub>-MSN, to exploit the aminopropyl  
13 functionality for covalent linking of the targeting agent. We followed three different procedures  
14 proposed in the literature [36, 37, 40]. The protocol described in Ref [40] was discarded since the  
15 prepared samples resulted in the lowest derivatization yield. This is probably ascribable to the low  
16 concentration of the coupling agent 1-ethyl-3-(3'-dimethylaminopropyl)carbodiimide (EDAC),  
17 notwithstanding the excess of HA with respect to MSN. The methodologies proposed by Yu et al.  
18 and by Ma et al. [36, 37], briefly described below, are hereafter mentioned as method A and B,  
19 respectively. HA of MW of 6.4 and 200 kDa were used ~~with each method~~ **for both preparations.**

20

#### 21 *2.3.1 Samples HA200A and HA6.4A*

22 This approach requires activation of HA through N-hydroxysuccinimide (NHS) and EDAC as  
23 coupling agent, before nanoparticles conjugation [37]. To this aim, 56 mg of NHS and 30 mg of  
24 EDAC were separately dissolved in 1.5 ml water, each. 17 mg of HA were suspended in 9 ml water,  
25 before adding the NHS and EDAC solutions previously prepared. The mixture was left under  
26 magnetic stirring at RT for 1 h. 150 mg of NH<sub>2</sub>-MSN were suspended by sonication in 15 ml of



1 water, before adding the activated HA solution. pH was then adjusted to 9 with trimethylamine  
2 (TEA). The mixture was heated to 38 °C and left under stirring overnight. After cooling down to  
3 RT, the supernatant was separated by centrifugation (60 rpm, 20 min). The solid powdered product  
4 was washed thrice with water; and suspended in few ml of water before freeze-drying. This  
5 procedure was carried out with HA 200kDa and 6.4 kDa, resulting in samples HA200A and  
6 HA6.4A, respectively.

7

### 8 *2.3.2 Samples HA200B and HA6.4B*

9 ~~With this method,~~ Following method B, activation of HA was carried out directly in the presence of  
10 the nanoparticles. 150 mg of NH<sub>2</sub>-MSN were sonicated in 30 ml of 2-(N-  
11 morpholino)ethanesulfonic acid (MES) buffer 0.01 M, at pH 6. 150 mg of HA were dissolved in 15  
12 ~~of ml~~ of MES and added to the nanoparticles suspension. 15 mg of EDAC and 15 mg of NHS, each  
13 dissolved in 7.5 ml of MES, ~~are~~ were added to the MSN suspension. The reaction was kept under  
14 magnetic stirring at RT for 4 h and successively centrifuged (60 rpm, 20 min). After removal of the  
15 supernatant, the solid powdered product was washed thrice with water and re-suspended in few ml  
16 of water for freeze-drying [36]. The procedure was carried out with HA 200kDa and 6.4kDa,  
17 resulting in samples HA200B and HA6.4B, respectively.

18

### 19 *2.3.3 Fluorescent labelling*

20 FITC labelled NH<sub>2</sub>-MSN and MSN/HA samples were prepared as reported in Ref. [37] with minor  
21 modifications. Briefly, 250 µl of FITC ethanol solution (0.3 mg/ml) were added at to a suspension  
22 of 1 mg of MSN in 150 µl of water ~~250 µl of FITC ethanol solution (0.3 mg/ml) were added.~~ The  
23 mixture was maintained at RT for 6 h under stirring in the dark and then the nanoparticles were  
24 centrifuged (60 rpm, 10 min) and washed with ethanol thrice until the supernatants were colorless.

25

### 26 *2.4 Physico-chemical characterization*

1 ~~High Resolution~~ Transmission Electron Microscopy (HRTEM) analyses were performed by means  
2 of measurements were carried out with a JEM 3010-UHR microscope (JEOL Ltd.) operating at 300  
3 kV. ~~For the measurements,~~ Powders were dispersed on a copper grid coated with a perforated  
4 carbon film. The size distribution of the samples was obtained by measuring a statistically  
5 representative number of particles (*ca.* 250 particles) and the The results are indicated as mean  
6 particle diameter (dm)  $\pm$  standard deviation (STD) (dm $\pm$ STD).

7 Specific surface area (SSA), cumulative pore volume and pore size distribution of samples were  
8 calculated by gas-volumetric analysis measuring N<sub>2</sub> adsorption-desorption isotherms at liquid  
9 nitrogen temperature (LNT) using an ASAP 2020 physisorption analyser (Micromeritics). The SSA  
10 was calculated by the Brunauer-Emmett-Teller (BET) method and the average pore size was  
11 determined by means of the Barrett-Joyner-Helenda (BJH) method, employing Kruk-Jaroniec-  
12 Sayari (KJS) equations on the adsorption branch of nitrogen isotherms. Before the measurement,  
13 the samples were outgassed at RT overnight.

14 Powder X Ray Diffraction (PXRD) patterns were collected with a PW3050/60 X'Pert PRO MPD  
15 diffractometer (Panalytical) working in Bragg-Brentano geometry, using Cu K $\alpha$  radiation (40 mA  
16 and 45 kV), with a scan speed of 0.0167° min<sup>-1</sup> and a measure time of 200 s/step. The measure was  
17 carried out at low angles, in the range of 1.5-12°.

18 Thermogravimetric analysis (TGA) was carried out on a Q600 analyzer (TA Instruments) heating  
19 the samples at a rate of 10 °C/min from RT to 1000°C in air flow. Before starting measurements,  
20 samples were equilibrated at 30°C.

21 Fourier Transform Infrared (FTIR) spectra were recorded using an IFS28 spectrometer (Bruker  
22 Optics) equipped with a MCT detector, working with a resolution of 4 cm<sup>-1</sup> over 64 scans. The  
23 spectra were obtained in transmission mode, with the samples pressed in the form of self-supporting  
24 pellets, mechanically protected with a pure gold frame. Samples were placed in quartz cells  
25 equipped with KBr windows, allowing *in situ* activation and measurement. Before spectra  
26 measurement the samples were outgassed at RT for 4 hours to remove adsorbed water and

1 impurities. Spectra were normalized with respect to pellet thickness for direct comparison, by using  
2 the silica overtone modes in the 2100-1500  $\text{cm}^{-1}$  interval.

3 Colorimetric carbazole test was carried following the procedure described in [47]. Briefly, a  
4 suspension of 4 mg/ml in water was prepared for each sample. Two aliquots (60 and 180  $\mu\text{l}$ ) were  
5 diluted to a final volume of 1 ml in water. 3 ml of a 0.025 M  $\text{Na}_2\text{B}_4\text{O}_7$  in  $\text{H}_2\text{SO}_4$  96% solution were  
6 added to each tube. The suspensions were then shaken and heated at 100°C for 10 min. After  
7 cooling down, 100  $\mu\text{l}$  of a carbazole solution 0.1% p/v in absolute ethanol was added, before  
8 mixing and heating again at 100°C for 10 min. After cooling down, absorbance was measured at  
9 530 nm using a DU-70 Beckman spectrophotometer.

10 Microgravimetric  $\text{H}_2\text{O}$  adsorption/desorption isotherms were measured with an intelligent  
11 gravimetric analyzer (IGA-002, Hiden Analytical), based on an ultrahigh-vacuum (UHV)  
12 microbalance (weighing resolution of 0.2  $\mu\text{g}$ ) with integrated temperature and pressure control.  
13 Temperature control was based on a thermostated water bath/circulator, while pressure control was  
14 achieved with a Baratron capacitance manometer (accuracy  $\pm 0.05$  mbar). Buoyancy corrections  
15 were carried out using the weights and densities of all the components of the sample (including  
16 adsorbed phase) and counterweight sides of the balance, and the measured temperature. The mass  
17 uptake was measured as a function of time, and the approach to equilibrium of the mass relaxation  
18 curve was monitored in real time using a computer algorithm (real time processor, RTP). For each  
19 isotherm point the time origin of real-time analysis was set at 75% of the pressure change, while the  
20 minimum and maximum data collection time were set to 5 and 60 mins, respectively. RTP uses last-  
21 squares regression of a linear driving force (LDF) model in order to extrapolate a value of the mass  
22 relaxation asymptote and assess the time-scale of interaction. The samples were loaded in a sealed  
23 stainless steel reactor, where they were outgassed at 50°C overnight prior to  $\text{H}_2\text{O}$  dosage, in order to  
24 measure the sample dry weight. Two consecutive adsorption/desorption isotherms were measured  
25 varying the water equilibrium pressure in the 0-20 mbar interval (step of 3 mbar) at 28 °C. This  
26 corresponds to a maximum  $p/p^0$  value around 0.55 ( $p^0 \cong 38$  mbar at the measurement temperature).

1 The experimental isotherms were analyzed with a Langmuir model, which assumes a monolayer  
2 adsorption onto the surface and is expressed by the equation:

$$q = \frac{q_m K p}{1 + K p} \quad (1)$$

3  
4 where  $q$  is the equilibrium water uptake,  $q_m$  is the adsorption capacity (corresponding to the  
5 monolayer saturation) and  $K$  the Langmuir constant, which corresponds to the adsorption  
6 equilibrium constant [48].  $q_m$  and  $K$  were calculated via the linearization of the Langmuir equation,  
7 as follows:

$$\frac{p}{q} = \frac{1}{K q_m} + \frac{p}{q_m} \quad (2)$$

8  
9 Calculations were carried out expressing the equilibrium pressure as  $p/p^0$ , so that the  $K$  values  
10 reported in Table 4 are dimensionless.

11 The mean hydrodynamic size was determined at 25°C by Dynamic Light Scattering (DLS) using a  
12 nanosizer (Nanosizer Nano Z, Malvern Inst., Malvern, UK). The selected angle was 173° and the  
13 measurement was ~~taken~~ carried out after dilution of the nanoparticle suspensions in water and in  
14 phosphate buffered saline (PBS 0.1 M, pH = 7.4). The particle surface charge was investigated by  
15 zeta potential measurements at 25°C in water and PBS solution applying the Smoluchowski  
16 equation and using the ~~Nanosizer Nanoseries~~ Zetasizer Nanoseries ZS 90 (Malvern Instruments-). In  
17 both cases, measurements were carried out in triplicate by diluting 80 µl of a 1mg/ml particles  
18 suspension in water with the selected medium, to reach a final volume of 1 ml.

19 For the dispersity test 2 mg of NH<sub>2</sub>-MSN and MSN/HA samples were added to 1 ml of different  
20 medium [PBS 0.1 M or Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% of  
21 fetal bovine serum (FBS)] and bath sonicated for 30 min. ~~then dispersity~~ The stability of the  
22 ~~dispersions was evaluated~~ after 0, 4, 8, 24 and 30 h ~~was evaluated~~.

23

24 *2.5. Tumour cell lines culture*

1 MDA-MB-231 (human breast adenocarcinoma) and A2780 (human ovarian carcinoma) cells were  
2 used. MDA-MB-231 cells were grown in DMEM supplemented with 10% of FBS, 0.03% of L-  
3 glutamine and 2% penicillin and streptomycin. A2780 cells were cultured in RPMI 1640 medium  
4 containing 10% FBS, 0.03% of L-glutamine, 2% penicillin and streptomycin, and 50 g/ml of  
5 gentamicin sulfate. Cells were maintained in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

## 6 7 *2.6. Receptor expression analysis*

8 Flow cytometry was used to determine the presence of CD44 on the cell surface by indirect  
9 immunofluorescence. Cells were washed twice with PBS and incubated 30 min in the dark at 4 °C  
10 with CD44 primary antibody, washed twice with PBS and then incubated 30 min in the dark at 4 °C  
11 with a phycoerythrin (PE) conjugated goat antibody (Dako Italia, Milan, Italy). Samples were  
12 analyzed on a flow cytometer instrument (FACSCanto, Becton Dickinson, San Jose, CA). Dead  
13 cells and debris were excluded on the basis of forward-scatter and side-scatter. Flow cytometry data  
14 were analyzed using the FlowJo software (TreeStar, Ashland, OR). CD44 expression was measured  
15 by calculating the ratio between median fluorescence intensity of cells labelled with antibodies  
16 versus unlabelled cells (Relative Median Fluorescence Intensity, RMFI).

## 17 18 *2.7. Incubation with MSN and cytotoxicity evaluation*

19 MDA-MB-231 and A2780 cells were seeded at  $1 \times 10^4$  cells/well in 96 wells microtiter plates and  
20 incubated overnight to allow cellular adhesion. Various dilutions of NH<sub>2</sub>-MSN and MSN/HA  
21 samples (1.5-25 µg/ml) were added in triplicate, and incubated for 24, 48 and 72 h.

22 Cell growth inhibition was evaluated by sulforhodamine B (SRB) colorimetric proliferation assay,  
23 modified by Vichai and Kirtikara [49].

## 24 25 *2.8 Confocal analysis of fluorescent MSN*

1 After 24 h incubation with either FITC labelled NH<sub>2</sub>-MSN or MSN/HA, MDA-MB-231 and A2780  
2 cells were fixed for fluorescence microscopy with 4% (v/v) paraformaldehyde in PBS, pH 7.4, for  
3 30 min at room temperature. The samples were stained for DNA with Hoechst 33342 (1 µg/ml in  
4 PBS for 5 min; Sigma), counterstained with 0.1% Trypan blue in PBS for 30 sec to visualize the  
5 cytoplasm, rinsed in PBS, and mounted in a 1:1 mixture of glycerol:PBS (Calbiochem, Inalco,  
6 Milan, Italy). For confocal laser scanning microscopy (CLSM), a Leica TCS SP5 AOBS system  
7 (Leica Microsystems Italia, Milan, Italy) was used with a 40x oil immersion objective. For  
8 fluorescence excitation, a diode laser at 405 nm for Hocheist, an Ar laser at 488 nm for FITC and a  
9 He/Ne laser at 543 for Trypan blue were employed. Z-stack of 1.5 µm step sized images (each  
10 image in the 1024x1024 pixel format) were collected and processed by the Leica confocal software.  
11 The RGB channels of the images presented here are the gray intensity images obtained with 405 nm  
12 (B), 488 nm (G) and 533 nm (R) excitation wavelengths respectively.

13

### 14 *2.9 Cellular uptake*

15 A quantitative determination of the cellular uptake was performed on a fluorescence-activated cell  
16 sorter (FACS). MDA-MB-231 and A2780 cells seeded in 6-well culture plates (5X10<sup>5</sup> cells/well),  
17 were exposed for different **length of** time (15 min, 30 min or 1 h) to FITC labelled NH<sub>2</sub>-MSN and  
18 MSN/HA with equivalent fluorescence. After removal of the free FITC-NH<sub>2</sub>-MSN and FITC-  
19 MSN/HA, cells were washed twice with PBS, collected by tripsynization and finally re-suspended  
20 in 1 ml of PBS. Then the intracellular uptake of MSNs was analyzed using a FACScan. Detection  
21 of FITC-MSNs green fluorescence (FL-1) was performed on at least 5,000 events per samples,  
22 using the CellQuest software (Becton-Dickinson, Milano, Italy). Cells incubated in the absence of  
23 MSN were used as control.

24

## 25 **3. Results and discussion**

1 ~~All samples were characterized about their general properties,~~ The general properties of all samples,  
2 including textural and morphological features, were analyzed. Sample HA6.4A was discarded due  
3 to low derivatization yield and to scarce dispersity (see later), and will thus not be described in  
4 detail in the following. The NH<sub>2</sub>-MSN sample used for HA conjugation was measured for  
5 comparison. Corresponding results are hereafter discussed only when relevant.

6

### 7 *3.1 Effect of conjugation on porous structure*

8 MSN/HA samples are composed by spherical nanoparticles (particle size 94±20 nm, see  
9 corresponding histogram as Figure S1), which are characterized by an ordered pore structure with  
10 the typical hexagonal array of MCM-41-like materials. This can be appreciated in the high  
11 resolution TEM picture of sample NH<sub>2</sub>-MSN, reported in Fig 1a, showing two superimposed  
12 particles with pores parallel and perpendicular to the image plane (circle and hexagon shapes,  
13 respectively). The same particle size, morphology and ordered porosity is preserved after HA  
14 conjugation, as shown in Figures 1b-d. However, in this case an amorphous-like external layer is  
15 clearly observed, which can be attributed to the formation of a shell of HA, covering the particles.  
16 The formation of a HA shell is supported by both qualitative and quantitative characterization  
17 results, described in the following.

18 The ordered pore structure of the materials is also reflected in the typical XRD pattern,  
19 characterized by low angle peaks at 2.3, 4.0 and 4.7° (Figure S2). These can be labelled as (100),  
20 (110) and (200) in the P6mm symmetry group, corresponding to the hexagonal pore array of  
21 pores. After HA conjugation, in all samples the peaks decrease in intensity and move to slightly  
22 higher 2θ values in all samples. The change is in the order HA200A < HA6.4B ≅ HA200B. Both  
23 phenomena are usually observed in MCM-41-like materials after functionalization and/or drug  
24 encapsulation, and are interpreted in terms of molecules filling/lining the pores [50, 51].  
25 Particularly, the d<sub>100</sub> parameter, which is the distance between planes passing through the pores

1 centre (see Figure S3), can be calculated from the Bragg equation, as resumed in Table 1. These  
2 data are further discussed hereafter in combination with results from gas-volumetric analysis.

3 The nitrogen adsorption/desorption isotherms of the three MSN/HA and parent NH<sub>2</sub>-MSN materials  
4 are reported in Figure 2 (top panel), with the corresponding pore size distribution calculated with  
5 the BJH method (bottom). Curves a) and b), corresponding to NH<sub>2</sub>-MSN and HA200A, can be  
6 classified as type IV, typical of mesoporous materials. Namely, a steep increase of the adsorbed  
7 amount is observed below  $p/p^0 = 0.3$ , which corresponds to the capillary condensation of the  
8 adsorbate inside the pores. The narrow hysteresis loop present in all samples at  $p/p^0$  reaching 1 is  
9 instead related to condensation of nitrogen in the interparticle porosity. After conjugation, the  
10 nitrogen adsorbed amount decreases in the order HA200A > HA6.4B  $\cong$  HA200B, and in the latter  
11 last two the capillary condensation related to mesopores filling is no more present. This is reflected  
12 in the corresponding pore size distribution curves (Figure 2, bottom panel). Parent NH<sub>2</sub>-MSN  
13 material shows an intense and relatively narrow peak centred around 30 Å, corresponding to the  
14 pores lined with aminopropyl groups. This peak is weaker and shifted to lower values for HA200A,  
15 very weak and not present for in HA6.4B and HA200B, respectively.

16 The textural parameters obtained from these data are summarized in Table 1. On the whole, we  
17 observe a major effect of conjugation on samples prepared by method B, where Specific Surface  
18 Area (SSA), pore volume and diameter are sensibly decreased. This suggests that during this  
19 procedure (where HA activation is carried out directly in the presence of the nanoparticles) the  
20 polymer is not only forming an external layer but it is also diffusing inside the pores. This is less  
21 likely in the case of high molecular weight HA, where the polymer could mainly block pores  
22 entrance not allowing nitrogen molecules to diffuse within during BET measurements. On the other  
23 hand, method A, where HA activation is carried out before reaction with NH<sub>2</sub>-MSN, results in a  
24 minor diffusion inside the pores, which are less obstructed to the diffusion of the nitrogen adsorbate  
25 used for gas-volumetric analysis. These considerations are in very good agreement with XRD and  
26 TEM observations, showing an apparently thicker HA layer on sample HA200B with respect to the



1 other two (compare Figure 1b with c and d). Finally, the mean pore diameter calculated by BJH  
2 method was combined with the  $a$  parameter calculated from XRD (distance between centres of the  
3 pores, see Figure S3), to calculate the apparent wall thickness. The values, summarized in last  
4 column of Table 1, clearly show an increase in the order HA200A < HA6.4B  $\cong$  HA200B, in  
5 agreement with the above considerations.

6

### 7 3.2 Quantitative HA analysis

8 Quantification of HA ~~bonded~~ covalently linked to the NH<sub>2</sub>-MSN particles was carried out by two  
9 independent techniques, namely TGA and the carbazole colorimetric test.

10 TGA was carried out from 30 to 1000 °C in air flow, in order to quantify the amount of organic  
11 groups anchored to the silica surface. This was calculated on the basis of the observed weight loss,  
12 normalized to the dry mass of the sample, *i.e.* after removal of physisorbed water around 100 °C  
13 (Figure S4). All MSN/HA samples show a consistent weight loss between *ca* 180 and 650 °C,  
14 which is higher with respect to parent NH<sub>2</sub>-MSN, in the order HA200A < HA200B  $\cong$  HA6.4B. The  
15 calculated amounts are summarized in Table 2. More details about the criteria used for  
16 quantification can be found in the Supplementary Material. In this context, it is sufficient to  
17 mention that samples HA200B and HA6.4B show very similar weight losses and curves slope,  
18 while HA200A has an intermediate profile, both in terms of quantity and curve shape, in very good  
19 agreement with the trends observed with textural and structural techniques, as described above.

20 Carbazole colorimetric test was also employed for HA quantification. As mentioned above, HA is a  
21 high molecular weight glycosaminoglycan, formed by a repeating disaccharide unit composed by D-  
22 glucuronic acid and *N*-acetyl-D-glucosamine. In this test HA is hydrolyzed in acid environment, so  
23 that monosaccharide units can form coloured adducts with carbazole through their glucuronic acid  
24 residues. This allows the quantification of HA through a simple optical measurement.

25 The values obtained with both techniques are resumed in Table 2, expressed as HA weight  
26 percentage. First of all, we underline the very good agreement between the two techniques.

1 Secondly, the results show that a definitely higher mass of HA has been linked to MSN with  
2 method B, irrespective of its molecular weight. Method A definitely yields a materials with lower  
3 (around one third) HA loading. This could be explained by the fact that in the latter case HA  
4 activation was carried out before reaction with NH<sub>2</sub>-MSN. On the basis of gas-volumetric and TEM  
5 results, we infer that this could result in a more difficult diffusion inside the pores, so that mainly  
6 external aminopropyl groups were involved in the reaction. Finally, these results contribute to the  
7 interpretation of the structural and textural parameters of the MSN/HA samples, which appear to be  
8 mainly affected by the HA loading, irrespective of its molecular weight.

9

### 10 *3.3 Qualitative HA analysis: infrared spectroscopy*

11 Infrared spectroscopy is a powerful tool to investigate interface interactions in hybrid organic-  
12 inorganic materials. It can be used to assess the molecular structure of surface grafted  
13 functionalizing groups, and the weak interactions taking place between silica surface and  
14 adsorbed/encapsulated drug molecules [28, 50-58]. The spectra of parent NH<sub>2</sub>-MSN and HA  
15 conjugated samples are reported in Figure 3, in the high and low frequency ranges (top and bottom  
16 panel, respectively). All samples were measured in transmission mode on self-supporting pellets,  
17 after RT evacuation necessary to remove adsorbed impurities and water, which would influence the  
18 spectral analysis (see for instance Refs. [28] and [57]). The spectrum of HA 200 kDa (measured in  
19 KBr) is reported **for comparison** in the Supplementary Material (Figure S5). The bands assignment  
20 discussed in the following is resumed in Table 3.

21 The spectrum of parent NH<sub>2</sub>-MSN sample, reported for comparison, is similar to what already  
22 reported and discussed in the literature [28, 57]. Namely, it is characterized by a broad signal in the  
23 high frequency region (top panel), which is characteristic of hydrogen bonding interactions among  
24 surface Si-OH groups (silanols) and between these and the grafted aminopropyl functionalities. The  
25 presence of the aminopropyl groups is testified by the ~~components~~ **bands** (superimposed to the  
26 broad band described above) at 3370 and 3300 cm<sup>-1</sup> (antisymmetric and symmetric NH<sub>2</sub> stretching

1 modes,  $\nu\text{NH}_2$ , respectively) and at  $2930/2870\text{ cm}^{-1}$  (antisymmetric and symmetric  $\nu\text{CH}_2$ ). The very  
2 weak peak at  $3734\text{ cm}^{-1}$  is related to hydrogen bonding acceptors and/or ~~geminal~~ geminal  $-\text{Si}(\text{OH})_2$   
3 silanols, which were not consumed by the grafting reaction [57]. The corresponding fingerprints of  
4 aminopropyl functional groups in the low frequency region (bottom panel of Figure 3) are the band  
5 at  $1595\text{ cm}^{-1}$  due to the  $\text{NH}_2$  bending mode ( $\delta\text{NH}_2$ ) and the weak  $\delta\text{CH}_2$  features between  $1500$  and  
6  $1320\text{ cm}^{-1}$ .

7 The spectra of MSN/HA samples show interesting differences with respect to parent materials.  
8 Namely, in the high frequency region an increase in the intensity and spectral breadth of the  
9 absorption between  $3700$  and  $2200\text{ cm}^{-1}$  is observed, in the order  $\text{HA200} < \text{HA200 B} \cong \text{HA6.4B}$ .  
10 This can be clearly related to the presence of an extended hydrogen bonding network, both among  
11 HA residues and between them and the silica surface. Indeed, the weak band at  $3734\text{ cm}^{-1}$  related to  
12 Si-OH groups is consumed, confirming the involvement of surface silanols in hydrogen bonding  
13 interactions with HA.

14 Moreover, weak ~~components~~ bands can be also observed between  $3200$  and  $3000\text{ cm}^{-1}$ , recalling the  
15 typical spectral shape of hydrogen-bonding carboxylic acids. This indicates that not all the D-  
16 glucuronic acid residues were involved in the formation of an amidic bond with the amino groups  
17 present on silica. In agreement with the structural, textural and quantitative data discussed above,  
18 the two samples prepared by method B are very similar and more affected by conjugation, at  
19 variance with sample HA200A, showing an intermediate behavior.

20 The same trend is observed in the low frequency range (Figure 3, bottom panel). More in detail,  
21 broad absorptions develop in the  $1700 - 1500\text{ cm}^{-1}$  and  $1450 - 1350\text{ cm}^{-1}$  ranges, with the same  
22 order of intensity described above, *i.e.*  $\text{HA200} < \text{HA200 B} \cong \text{HA6.4B}$ . The  $\delta\text{NH}_2$  signal at  $1595\text{ cm}^{-1}$   
23 <sup>1</sup> is clearly evident on sample HA200A, and as ~~and as~~ while it is only a shoulder in the other samples. This  
24 indicates that not all aminopropyl functionalities reacted with HA, as expected for functional groups  
25 lining the inner surface of the pores. Interestingly, the intensity of this band is higher on sample

1 HA200A, where a lower diffusion of HA residues inside the pores was inferred from textural and  
2 structural analysis (see above).

3 Concerning the new ~~spectral~~ features developing in this spectral region after HA conjugation, they  
4 can be safely ascribed to the formation of an amidic bond between the NH<sub>2</sub>-MSN amino groups and  
5 the carboxylic acid functionalities of HA. Namely, the amide I band ( $\nu$ CO) is observed at 1650 cm<sup>-1</sup>,  
6 the amide II ( $\delta$ NH and  $\nu$ CN combination mode) at 1550 cm<sup>-1</sup>, while the absorption between 1430  
7 and 1350 cm<sup>-1</sup> is related to the numerous  $\delta$ CH and  $\delta$ OH vibrations of HA.

8

### 9 *3.4 Interaction with water molecules*

10 HA is a hydrophilic macromolecule, with interesting applications as wetting agent [59-61] or as  
11 polymer for surfaces modification. Indeed, recent studies reported about the use of HA to improve  
12 hydrophilicity and biocompatibility of chitosan films or scaffolds [62, 63]. Moreover, HA has been  
13 used to increase surfaces hydrophilicity to provide antifouling properties, as a result of reduced  
14 nonspecific protein adsorption [64-68]. In this work, the effect of HA conjugation on the  
15 hydrophilic character of MSN has been studied by carrying out water vapour sorption  
16 microgravimetric experiments. This technique has been recently employed by some of us to  
17 investigate the effect of different functional groups on the hydrophilic character of MSN [28].

18 Water adsorption/desorption isotherms at 28 °C were measured on the three MSN/HA, by gradually  
19 increasing the equilibrium vapour pressure in the 0- 20 mbar range ( $p/p^0$  from 0 to ca 0.55). The  
20 results obtained on sample HA6.4B are reported in Figure 4 (top panel). Similar trends were  
21 observed on samples HA200A and HA200B (see Figure S6) and can be summarized as follows.  
22 Both primary and secondary adsorption/desorption cycles are characterized by a hysteresis loop,  
23 with higher water uptake measured during the desorption step, for each  $p/p^0$  value. This can be  
24 explained by the slow diffusion of water interacting with the external HA layer surrounding the  
25 MSN. This implies that the equilibrium is probably not reached in the adsorption step within the  
26 time frame of the experiment (timeout of 60 minutes for each  $p/p^0$  dosage). A small water amount is

1 not desorbed after the first desorption step, and in all samples the second cycle results in a slightly  
2 higher water uptake. When comparing the secondary desorption curves on the three MSN/HA  
3 samples, the following order of water uptake is observed: HA6.4B > HA200A  $\cong$  HA200B (Figure  
4 4, bottom panel).

5 The obtained results are at first sight surprising, when compared to what measured on the parent  
6 NH<sub>2</sub>-MSN material, where a maximum uptake of 6.7 wt% (3.7 mmol/g) was measured at p/p<sup>0</sup>  $\cong$  0.3  
7 and 25 °C [28]. This is sensibly higher with respect to what obtained on the MSN/HA samples (1.3-  
8 1.7 wt%, see Table 4), indicating a decrease of in the water uptake of MSN after HA conjugation, in  
9 contrast to what expected for the high hydrophilicity of the polysaccharide. However, the  
10 explanation of this apparently puzzling results can be found through a more detailed analysis of the  
11 measured isotherms. To this aim, the primary and secondary desorption curves of the samples have  
12 been fitted with a Langmuir model, assuming that they are closer to the thermodynamic equilibrium  
13 with respect to adsorption ones. The results from this analysis are summarized in Table 4, together  
14 with the results for NH<sub>2</sub>-MSN parent sample (data from Ref. [28]). First, we acknowledge the fact  
15 that for all HA conjugated samples the agreement with the Langmuir model is satisfactory ( $R^2 >$   
16 0.99), which is not the case for NH<sub>2</sub>-MSN ( $R^2 = 0.9267$  for both cycles). Secondly, the set of  
17 calculated K values (corresponding to the equilibrium constant of the adsorption process) are  
18 consistent for the set of samples, ranging from 17 to *ca* 23, while for NH<sub>2</sub>-MSN a value around 8.5  
19 was found for both sorption cycles. These data clearly indicates that on the conjugated samples  
20 water is mainly interacting with the external HA layer, where it is strongly adsorbed and cannot  
21 freely diffuse inside the MSN pores, at least in the time frame and experimental conditions of the  
22 experiment. This picture fits well with the higher K values and smaller uptake with respect to parent  
23 NH<sub>2</sub>-MSN.

24 Coming back to the comparison among the three samples (Figure 4, bottom panel) the calculated K  
25 values are in the order HA200A < HA200B < HA6.4B. The last samples being the one with the  
26 highest uptake, we can infer that the polysaccharide molecular weight influences its hydrophilicity.

1 On the contrary, water uptake on HA200A is almost identical to HA200B, irrespective of the  
2 different loading. This supports the idea that, when dosed from vapour phase, water is strongly  
3 adsorbed on the external HA layer with limited diffusion within the pores. This effect could limit  
4 the diffusion of drugs to and from the material pores, as indicated by preliminary studies on drug  
5 loading carried out in our group (not reported). Noticeably, samples HA200B and HA6.4B shows a  
6 similar water/HA weight ratio (ranging from 0.07 to 0.09) with respect to the sample prepared by  
7 method A (0.21-0.22). These data further support the picture obtained by characterization  
8 techniques, indicating that sample HA200A is characterized by a different distribution of HA at the  
9 pores entrance, with a consistent amount of unreacted amino groups. This could indicate an easier  
10 diffusion of adsorbates (such as water molecules) and host drug molecules within the sample.

11

### 12 *3.3 Effect of HA on surface charge, aggregation and dispersity*

13 The electrophoretic mobility of the materials was measured to evaluate the effect of conjugation on  
14 the particles  $\zeta$  potential, which gives an indirect information about the surface charge in the Stern  
15 layer. This parameter is affected by both the hydrodynamic diameter and the electric double layer  
16 thickness, the latter being strongly influenced by the fluid ionic strength and pH. An estimation of  
17 the double layer thickness as a function of the ionic strength can be used in the Henry equation, to  
18 convert electrophoretic mobility into  $\zeta$  potential values [69]. However, in this work  $\zeta$  potential  
19 values (summarized in Table 2) were calculated with the Smoluchowski equation (representing an  
20 approximation valid for thick double layers), for direct comparison with the literature works in the  
21 field [28, 70-73].

22 In aqueous solution, the amine group is positively charged while the carboxylic ones of HA show a  
23 negative charge in relation to their pKa. Assessing the pKa of both covalently linked HA external  
24 layer and aminopropyl groups is not straightforward, since both systems are intrinsically complex.  
25 Indeed the pKa of amino-functionalized hybrid materials is **has been** the subject of extensive  
26 research work, since the covalent bond and interactions with the inorganic surface can affect the

1 amino basicity [28, 57, 74-78]. In our work,  $\zeta$  potential values in water change depending on the  
2 amount and on the molecular weight of the conjugated HA. At equal HA molecular weight  
3 (compare HA200A and HA200B, 2<sup>nd</sup> and 3<sup>rd</sup> lines in Table 2), the  $\zeta$  potential decreases as the  
4 amount of HA increases. Namely, HA200A (HA loading 6.6 wt%) shows a small but positive value  
5 ( $+14.3 \pm 1.3$  mV), while HA200B, with a HA loading almost triple is characterized by a negative  $\zeta$   
6 potential ( $-19.8 \pm 1.9$  mV). Samples obtained by method B showed a more negative surface charge;  
7 in fact, according to TGA and carbazole test, this method allows a more efficient conjugation.  
8 Considering the different HA molecular weights of samples obtained by the same conjugation  
9 method, HA200B showed a more negative surface charge with respect to HA6.4B, without clear  
10 correlation with the HA loading. Measurements were also carried out on sample HA6.4A, which  
11 was discarded for a more detailed characterization because of low HA loading (3.1 wt% as  
12 measured by carbazole test, see Table 2) and scarce dispersity (see below). Indeed, this sample  
13 shows a  $\zeta$  potential value in water almost identical to NH<sub>2</sub>-MSN, in agreement with the low  
14 derivatization yield.

15 When the same measurements are carried out in PBS, a different trend is observed, in that almost  
16 all samples show more negative  $\zeta$  potential values. As recently reported, buffer ions can strongly  
17 affect adsorption phenomena at the solid-liquid interphase of charged nanoparticles [73, 79, 80].  
18 Marucco *et al.* pointed out how adsorption of phosphate ions from the buffer solution can influence  
19 the particles surface properties and charge [79]. On the other hand, we cannot exclude the  
20 competition of chloride (and Na<sup>+</sup> and K<sup>+</sup> cations, although at a lesser extent) in modifying the  
21 effective surface charge of MSNs, in agreement with the work by Cugia *et al.* [73]. Indeed, in our  
22 work all the three considered HA conjugated samples show a similar negative  $\zeta$  potential value  
23 (around -16 mV), irrespective of the values measured in water. On the contrary, parent NH<sub>2</sub>-MSN  
24 still shows a positive (though minor) charge and sample HA6.4A is almost neutral ( $-1.3 \pm 0.5$  mV).

1 These results cannot easily be rationalized due to the complex interactions of buffer ions with  
2 nanoparticles [73].

3 DLS measurements were carried out to estimate the hydrodynamic sizes of all samples in both  
4 water and PBS (Table 2). The average values are always higher than the size of the single particles  
5 measured by TEM, indicating agglomeration. A slight decrease in the size is observed after HA  
6 conjugation, particularly for high molecular weight and loading, indicating a positive effect of the  
7 polymer against the MSNs tendency to agglomeration. Since our attention is focused on the  
8 possibility to use these systems for pharmaceutical application, we have followed the evolution of  
9 the hydrodynamic size with time. Figure 5 shows the results obtained on the more stable samples in  
10 PBS, *i.e.* HA6.4B, HA200A and HA200B. All The samples show a slight increase of dimensions in  
11 the first hours, and then reach a stable value, apart from sample HA6.4B which is characterized by  
12 larger size and lower stability. These observations are reflected in the dispersity tests described  
13 below.

14 The pictures acquired during dispersity tests in PBS are shown in Figure 6. In this case a higher  
15 concentration was used (2 mg/ml vs 0.08 mg/ml used for both  $\zeta$  potential and DLS measurements),  
16 for easier visual observation. The samples with HA of higher molecular weight were better  
17 dispersed with respect to parent NH<sub>2</sub>-MSN and HA6.4B, which started to precipitate after 4 h. On  
18 the contrary, dispersity was dramatically improved after conjugation with high molecular weight  
19 HA: HA200A and HA200B formed a stable suspension in PBS for more than 24 h. The enhanced  
20 stability of these samples can be explained on the basis of their steric hindrance and of the  
21 electrostatic repulsion among the stretched hydrophilic HA chains, reducing the possibility of  
22 agglomeration and precipitation. Finally, as concern sample HA6.4A, its precipitation is already  
23 complete already after 4 hours, in agreement with a  $\zeta$  potential value close to zero measured almost  
24 zero-charge. The dispersity tests carried out in DMEM +10% FBS showed a similar trend (data not  
25 reported).



1 These data confirm the working hypothesis that HA conjugation is a simple and effective method  
2 for improving the dispersity and stability of the nanoparticles, which is in turn beneficial for blood  
3 circulation making ~~the further~~ intravenous injection possible. Moreover, our study points out how,  
4 irrespective of the similar measured  $\zeta$  potential, which is influenced by the chemical nature of the  
5 buffer medium, the molecular weight of the conjugating macromolecule has a strong influence on  
6 dispersity.

7

### 8 *3.4 Biological characterization*

#### 9 *3.4.1. Analysis of cell surface CD44 expression*

10 The HA receptor CD44, an ubiquitous transmembrane molecule, is expressed at low levels on the  
11 surface of several normal cells and overexpressed in many cancer cells [81]. The levels of receptor  
12 cell surface expression were preliminary evaluated on a panel of cancer cell lines (MDA-MB-231,  
13 JR8, A459, MCF7 and A2780) using anti-CD44 antibody and flow cytometry, with the aim to  
14 identify the cell models to be used for further *in vitro* studies. The results show that A2780 cells did  
15 not express detectable amount of CD44 whereas MDA-MB-231 cells display a very high  
16 expression (Figure 7). Thus, to evaluate the cytotoxic activity and the cellular uptake of the  
17 nanoparticles, MDA-MB-231 and A2780 cells were chosen as CD44<sup>+</sup> and CD44<sup>-</sup> cancer cells,  
18 respectively.

19

#### 20 *3.4.2 In vitro cytotoxicity*

21 The *in vitro* cytotoxicity of NH<sub>2</sub>-MSN and MSN/HA was evaluated after treatment of 24, 48 and 72  
22 h in the concentration range of 1.5-25  $\mu\text{g/ml}$ . ~~and~~ The results showed that the nanoparticles were  
23 non-toxic for both cell lines demonstrating the good safety and biocompatibility of the carriers (data  
24 not shown). These data are in agreement with the results obtained with other HA decorated MSN  
25 [24, 36, 37, 40].

26

### 1 3.4.3 *In vitro* targeting analysis

2 Efficient cellular uptake is a major requirement for the therapeutic efficacy of nanoparticles  
3 targeting. To test the targeting ability of our MSN we evaluated the cellular uptake of both FITC-  
4 labelled NH<sub>2</sub>-MSN and MSN/HA on MDA-MB-231 (CD44+) and A2780 (CD44-) cell lines by  
5 both CLSM and FACS.

6 CLSM allowed **us to visualize** ~~visualizing~~ the cellular uptake and biodistribution of the  
7 nanoparticles ~~by~~ **in** these two cell lines. As shown in Figure 8, in both cell lines MSN occurred in  
8 the cytoplasm as clusters of different size; accordingly, previous **TEM** studies ~~at transmission~~  
9 ~~electron microscopy~~ demonstrated that these nanoparticles are internalized by the cell via both  
10 endocytosis and phagocytosis, and then accumulate into cytoplasmic vacuoles [82]. Moreover,  
11 MSNs were never found inside the nucleus of MDA-MB-231 and A2780 cells, consistently with  
12 previous observations ~~in the same or in different cell lines treated with these nanoparticles~~ [23, 24,  
13 40, 82-85]. Although CLSM does not allow precise quantitation of cellular uptake, the amount of  
14 internalized NH<sub>2</sub>-MSN was apparently similar in the two cell lines, whereas HA6.4B and HA200B  
15 were more abundant in MDA-MB-231 than in A2780 cells. This suggests that MSN/HA possess  
16 significant selectivity for cells overexpressing CD44 receptors, ~~consistently~~ **in agreement** with our  
17 results obtained with flow cytometry (see below).

18 In addition, microscopic observation of the samples revealed the absence of evident morphological  
19 alterations in both cytoplasm (e.g. vacuolization) and nucleus (e.g. pyknosis, apoptosis), thus  
20 confirming the high biocompatibility of NH<sub>2</sub>-MSN and MSN/HA at the concentrations tested in this  
21 study.

22 The morphological images obtained by CLSM are consistent with the flow cytometry analysis  
23 performed in order to obtain a quantitative comparison between FITC labelled NH<sub>2</sub>-MSN and  
24 MSN/HA ~~nanoparticles characterized by different preparation methods and different HA molecular~~  
25 ~~weight~~ (Fig. 9). After treating A2780 and MDA-MB-231 cells with the different MSN/HA, their  
26 uptake in A2780 cells is moderately increased in a time **dependent** ~~depending~~ manner, but almost

1 irrelevant in comparison with MDA-MB-231 (Fig. 9A). In particular, the FITC mean intensity of  
2 MSN/HA200 (both A and B) was dramatically increased in MDA-MB-231 in comparison to A2780  
3 (Fig. 9A, B). Concerning MDA-MB-231, we observed that the cellular uptake efficiency is  
4 increased over incubation time (up to 1h, when the uptake is near plateau, approximately 80%, Fig.  
5 9C). Moreover, the cellular uptake efficiency is also dependent on the amount of HA on  
6 nanoparticles surface and on its molecular weight. In particular, we obtained a higher uptake of  
7 HA200B versus HA200A, demonstrating that ~~the~~ method B in which the activation of HA was  
8 carried out directly in the presence of the nanoparticles, improves cells uptake (Fig. 9B). In  
9 contrast, the mean intensity of FITC from the cells incubated with FITC labelled NH<sub>2</sub>-MSNs  
10 without HA modification is lower and similar for both cell lines. These *in vitro* experiments  
11 confirm that MSN/HA can target CD44 over-expressing MDA-MB-231 cancer cells via the HA  
12 receptor-mediated endocytosis [84, 86] and phagocytosis [87] pathways, strongly highlighting the  
13 potential use of MSNs/HA as ~~more~~ **an** efficient approach for tumor-targeting treatments.

14

## 15 **Conclusions**

16 MSN/HA samples were prepared following two different conjugation procedures previously  
17 reported [36, 37] and using HA of two different molecular weight (6.4 and 200 kDa). The size of  
18 HA strongly affects its biological functions and its physico-chemical characteristics [35]. However,  
19 to our knowledge this is the first time that such a comparative study is reported, helping the  
20 scientific community working in the field to select the best strategy to obtain hybrid nanomaterials  
21 with good biological response and potential pharmaceutical applicability. More in detail, our  
22 systematic physico-chemical analysis points to the concerted effect of both HA molecular weight  
23 and loading ~~on MSN~~ in improving dispersity **of MSN**. With this respect, we have demonstrated that  
24 the ‘one pot’ method - in which HA is directly activated in the presence of the nanoparticles – is the  
25 most efficient, also in terms of possible scale-up.

1 Our results are also in agreement with recent reports about the importance of considering the  
2 interaction of buffer ions with nanoparticles when assessing surface charge and related interphase  
3 phenomena, which are of fundamental importance to rationalize results in physiological media [73].  
4 Finally, *in vitro* tests on cancer cell lines demonstrated that MSN/HA are biocompatible and  
5 preferentially target cells overexpressing the HA receptor CD44. Also in this context, the best  
6 performances are obtained with the ‘one pot’ sample prepared using HA with high molecular  
7 weight (HA200B). All these data suggest the potential use of MSNs/HA as a ~~more~~ efficient  
8 approach for tumor-targeting treatments.

9

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13 from Department of Chemistry and NIS Centre, University of Torino, for TEM measurements.

14

1 **Figure captions**

2 **Fig. 1** HRTEM images of a) NH<sub>2</sub>-MSN; b) HA200A; c) HA200B and d) HA6.4B

3

4 **Fig. 2** Nitrogen gas-volumetric adsorption (crosses) and desorption (squares) isotherms (top) and  
5 pore size distributions (bottom) of a) NH<sub>2</sub>-MSN; b) HA200A; c) HA200B and d) HA6.4B

6

7 **Fig. 3** Infrared spectra in the high and low frequency regions (top and bottom panels, respectively)  
8 of NH<sub>2</sub>-MSN (black); HA200A (blue); HA200B (red) and HA6.4B (green). Spectra were measured  
9 on dehydrated samples and are normalized with respect to pellet thickness

10

11 **Fig. 4** Water microgravimetric isotherms measured at 28 °C on MSN/HA samples. Top: primary  
12 and secondary adsorption/desorption measurements on sample HA6.4B; bottom: comparison of  
13 secondary desorption measurements on HA200A (◄), HA200B (■) and HA6.4B (●).

14

15 **Fig. 5** Dynamic light scattering data of HA200A (blue); HA200B (red) and HA6.4B (green) in PBS  
16 measured at 0, 4, 8, 24 and 30 h. The error bars represent the standard deviation of three  
17 measurements.

18

19 **Fig. 6** Images of NH<sub>2</sub>-MSN, HA6.4A, HA200A, HA6.4B and HA200B dispersed in PBS with a  
20 concentration of 2 mg/ml measured at 0, 4, 8, 24 and 30 h.

21

22 **Fig. 7** Flow cytometric histograms of CD44 expression in A2780 cells and MDA-MB-231 cells.  
23 Red lines: anti-CD44 antibody; blue lines: isotype control.

24

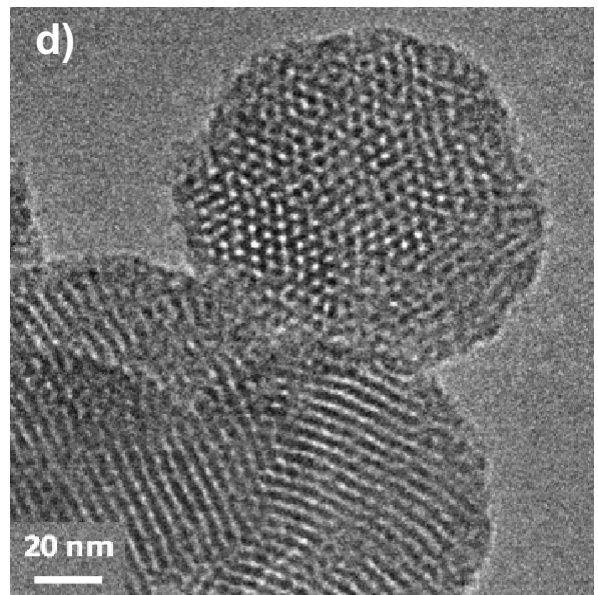
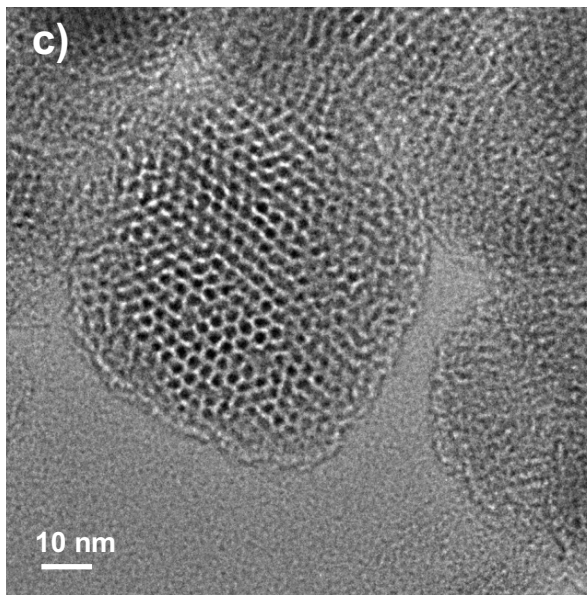
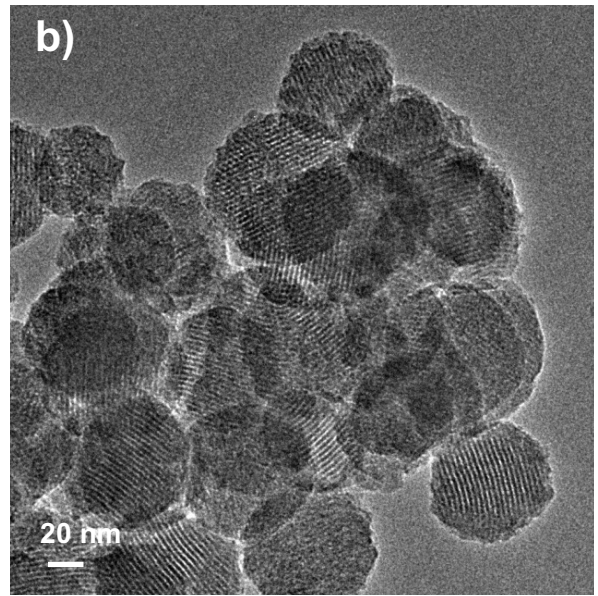
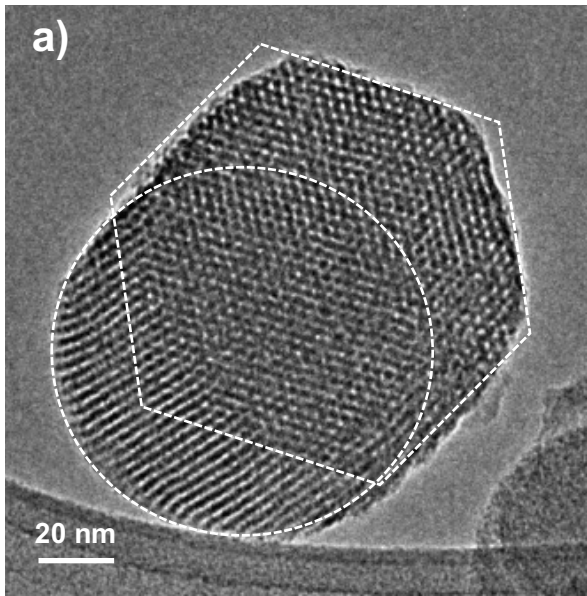
25 **Fig. 8** Confocal micrographs of A2780 cells (a-c) and MDA-MB-231 cells (d-f) after 24h  
26 incubation with different MSN (green fluorescence): NH<sub>2</sub>-MSN (a and d), HA6.4B (b and e) and

1 HA200B (c and f). All MSN are distributed in the cytoplasm but are absent from the nucleus (blue  
2 fluorescence). The cytoplasm is counterstained with trypan blue (red fluorescence). Note the higher  
3 amount of MSN in e and f. Bars: 10  $\mu$ m.

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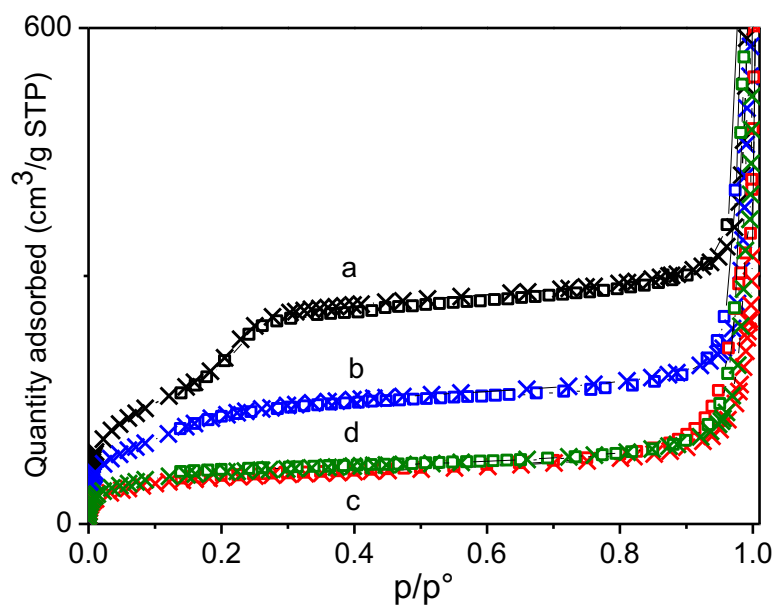
5 **Fig. 9** Cellular uptake of FITC labelled NH<sub>2</sub>-MSN with or without HA modification in A2780 and  
6 MDA-MB-321 cell lines. Analysis of internalization of NH<sub>2</sub>-MSN, HA6.4B, HA200A and  
7 HA200B in A2780 (A) and MDA-MB-231 (B,C) cells by flow cytometry for the indicated time.  
8 Data in graphs are expressed as means  $\pm$  SEM (\*p< 0.05 and \*\* p<0.01 vs FITC-NH<sub>2</sub>-MSN) of  
9 three independent experiments.

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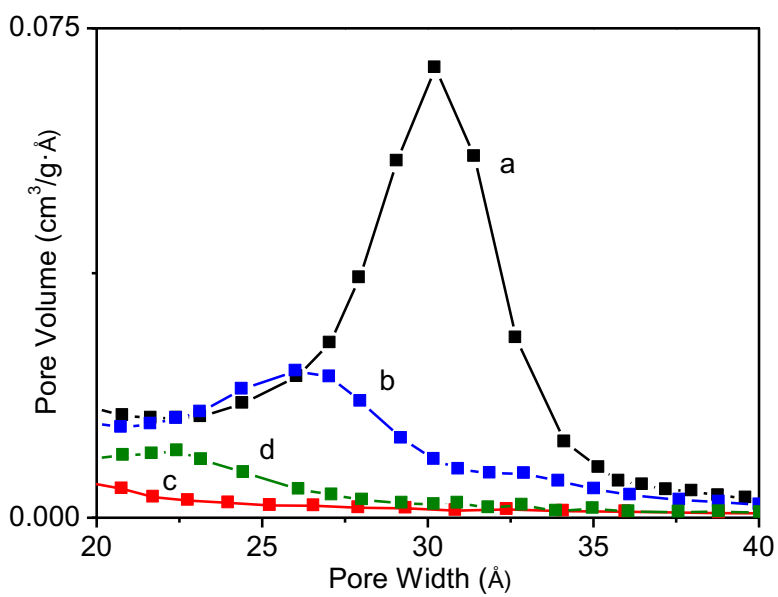


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**Fig. 1**



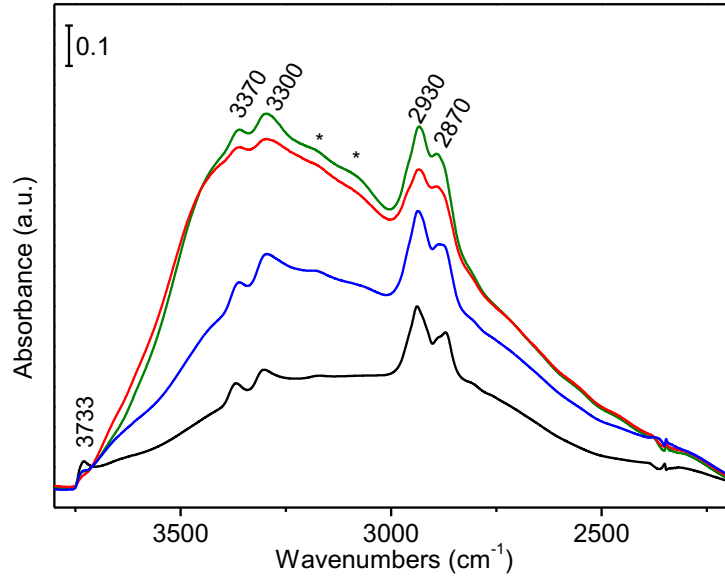
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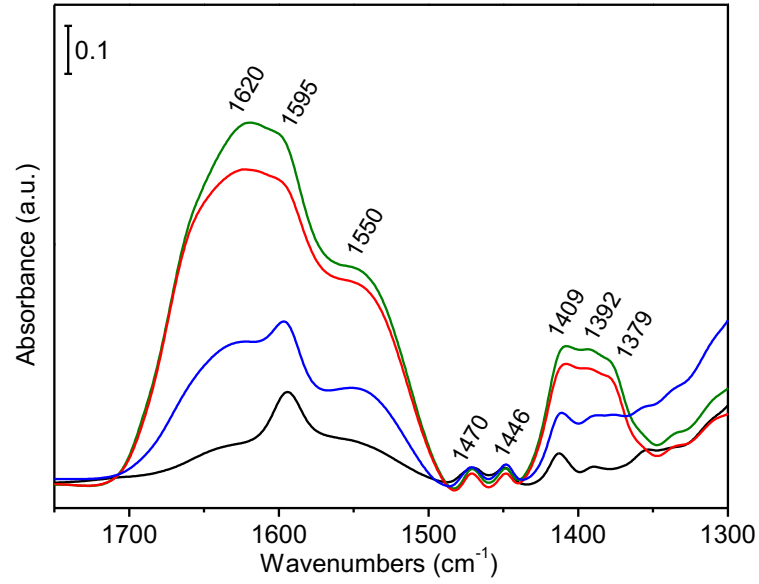
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Fig. 2





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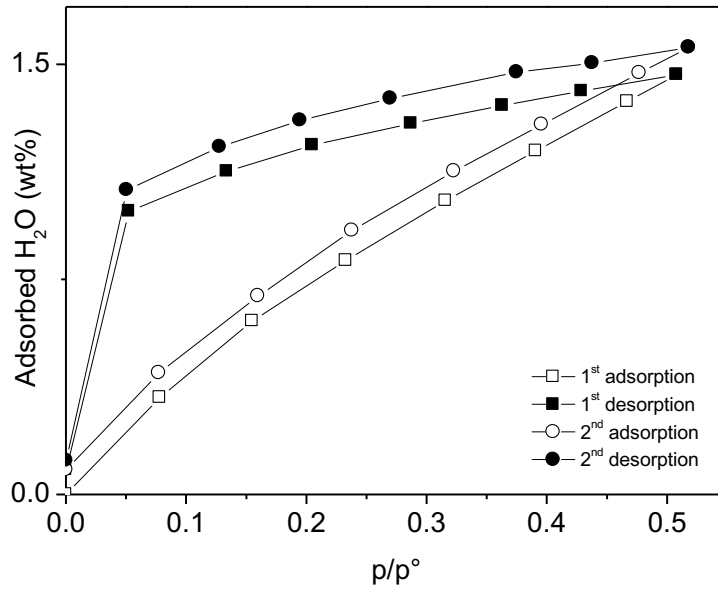


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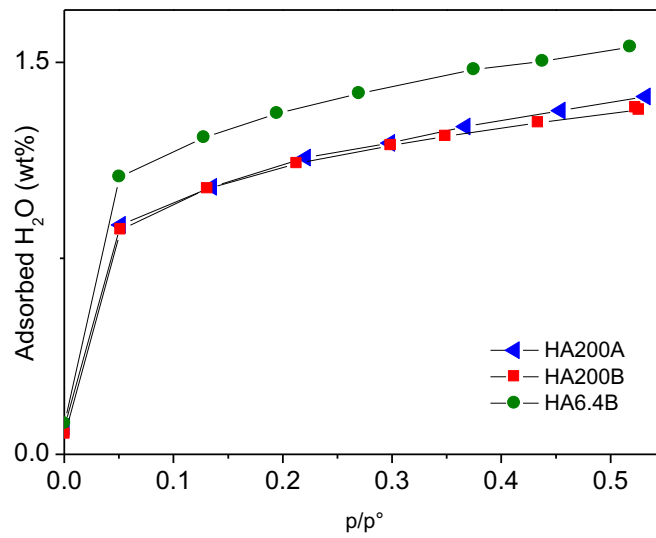
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**Fig. 3**

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Fig. 4

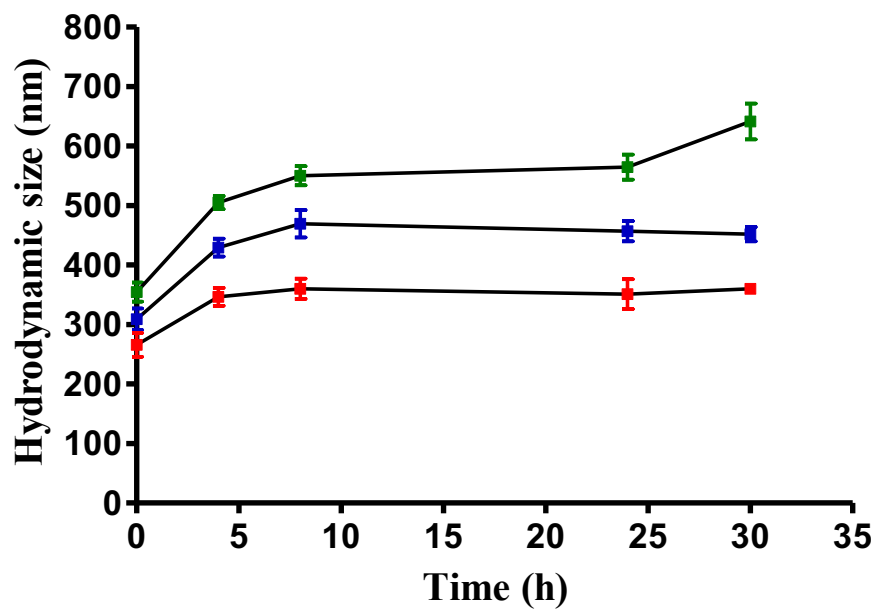


Fig. 5

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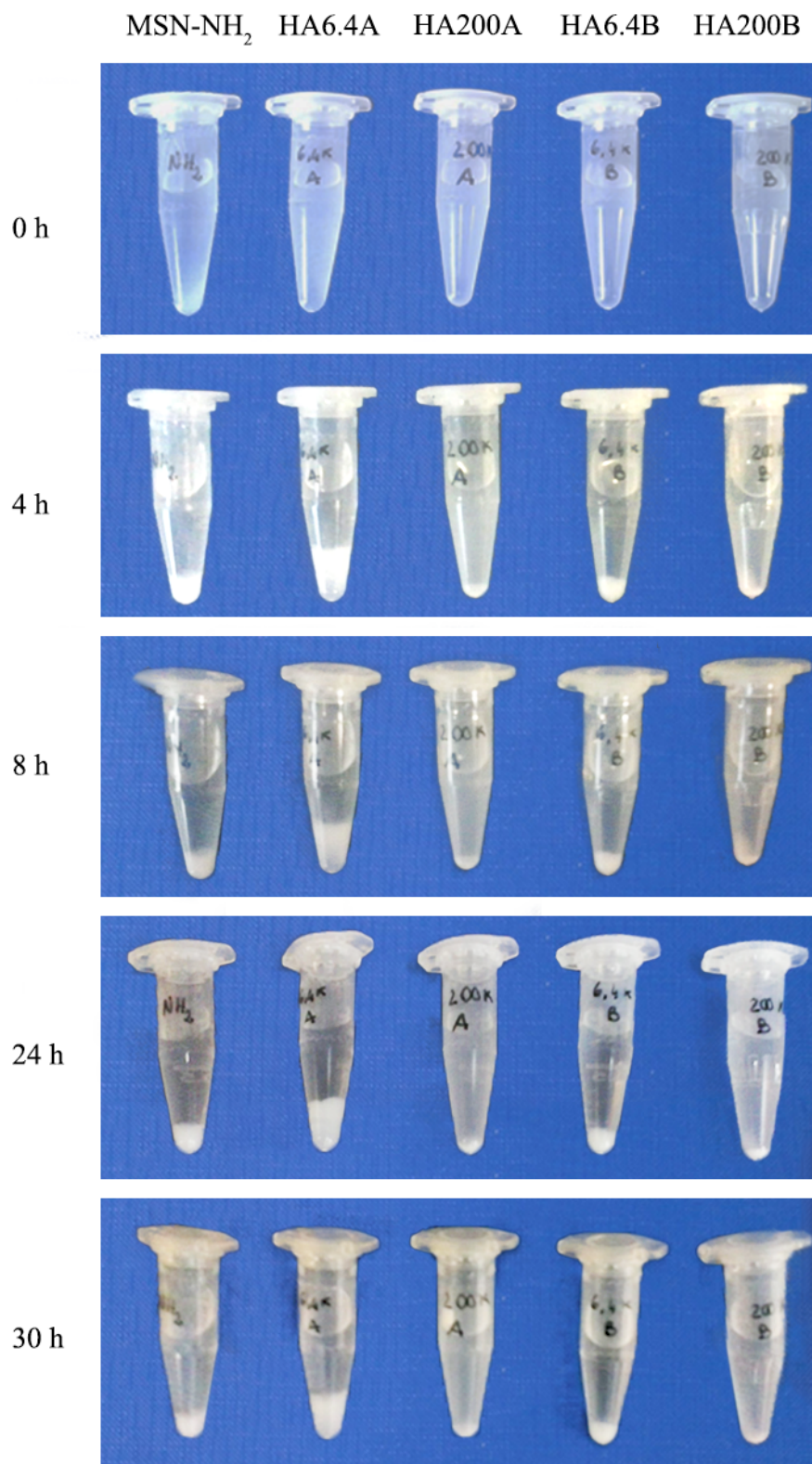
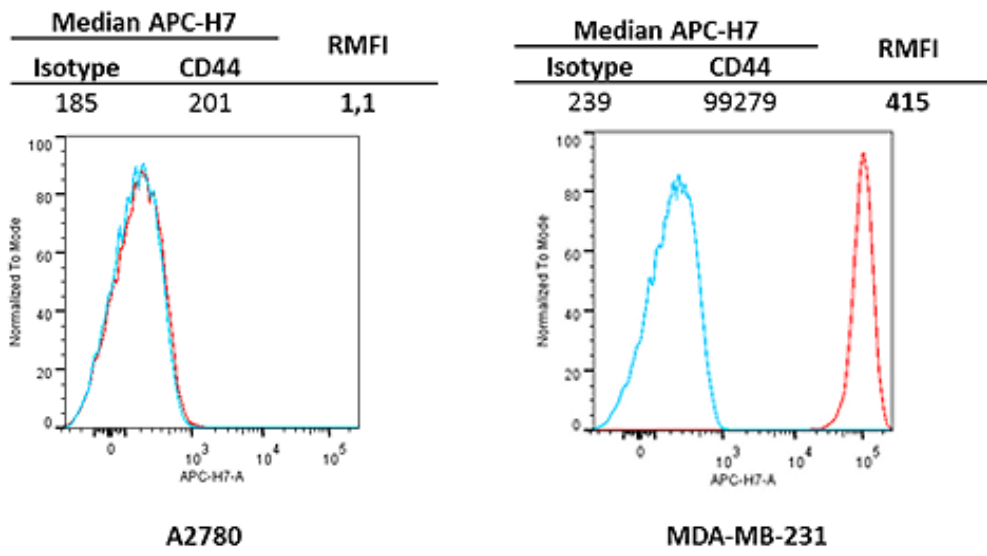


Fig. 6

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Fig. 7

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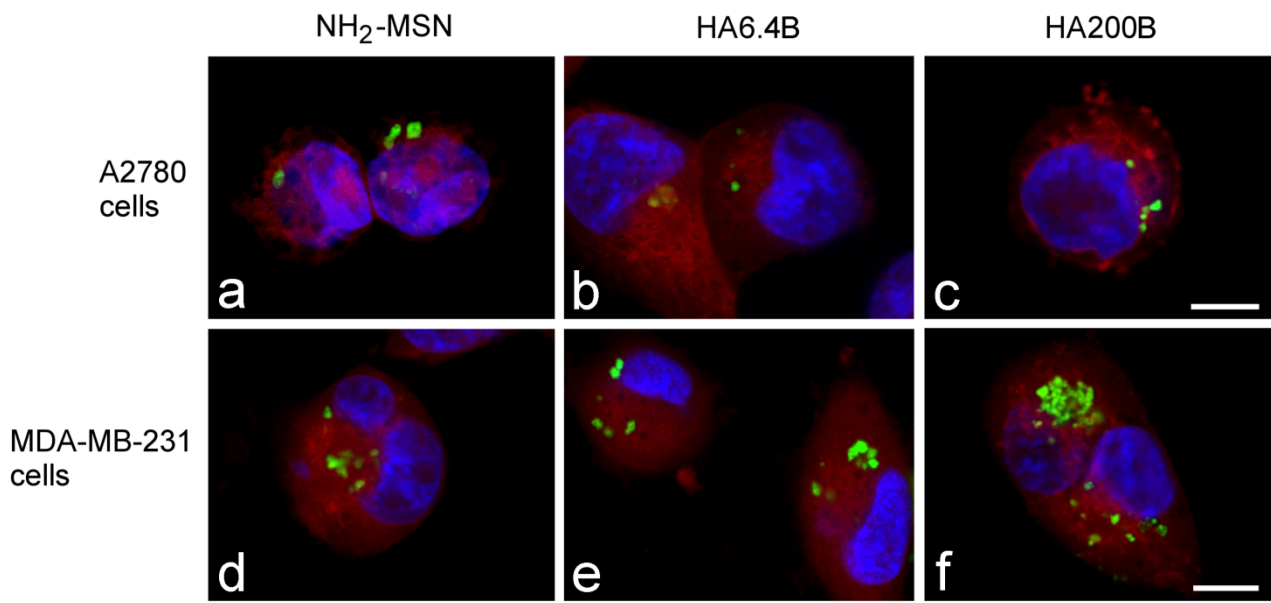
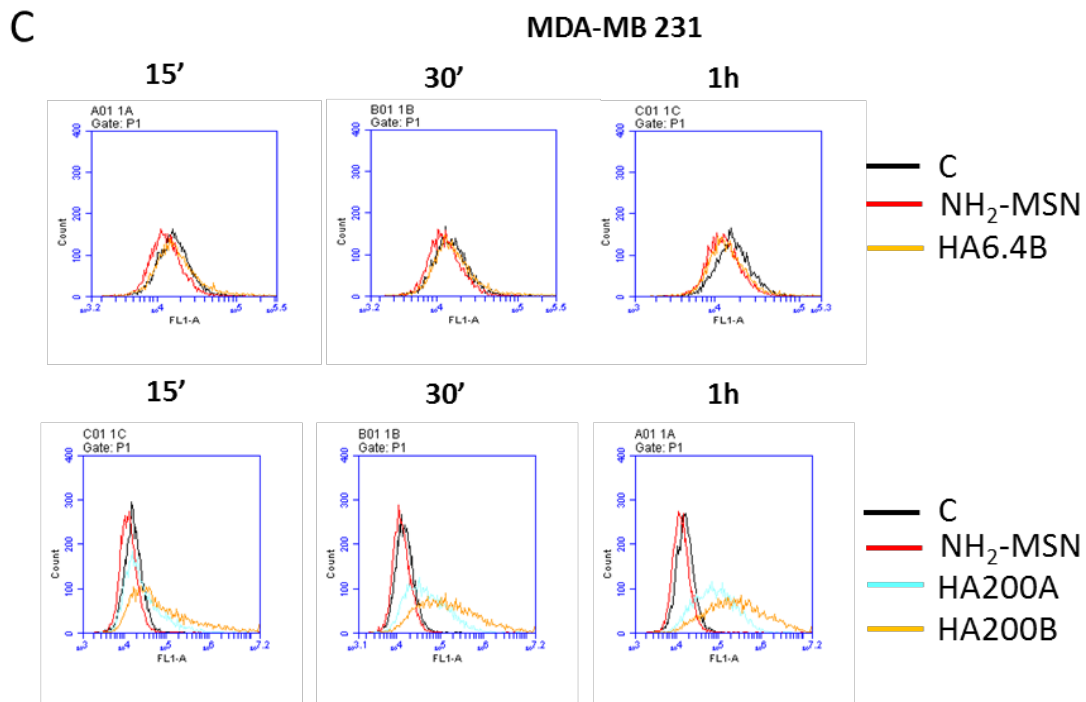
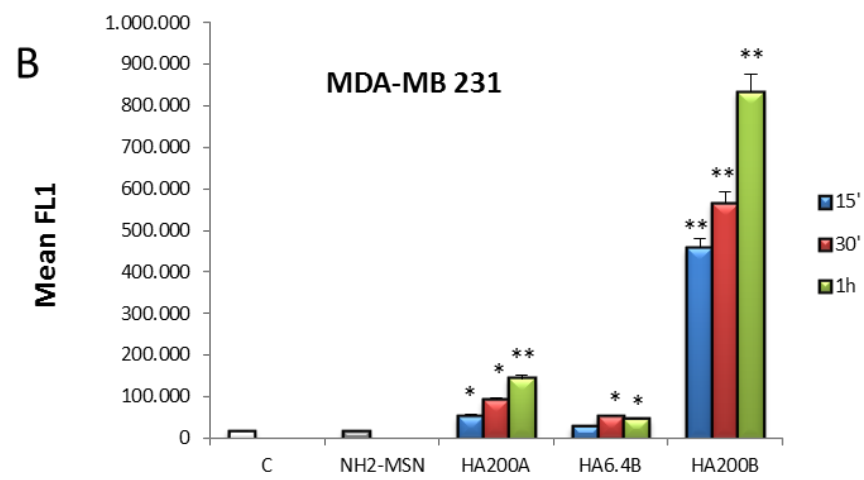
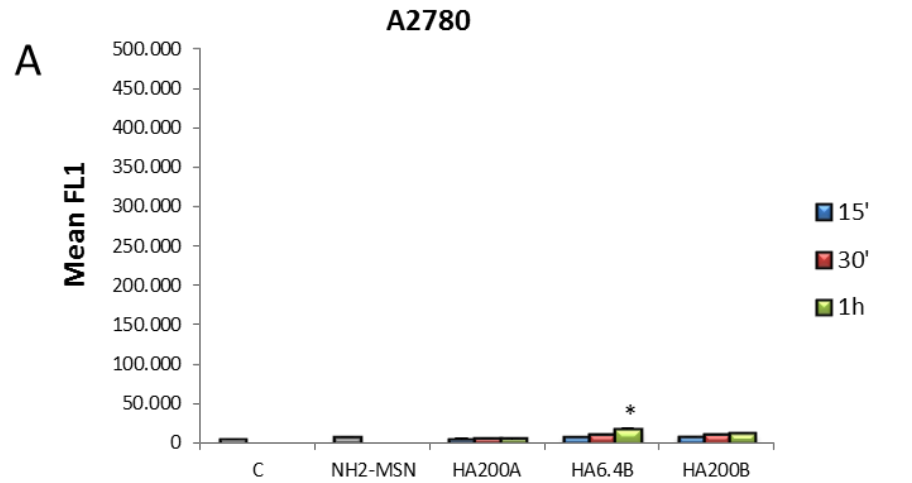


Fig. 8

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**Fig. 9**

1 **Tables**

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**Table 1:** Textural and structural properties of the studied materials

Samples	SSA (m <sup>2</sup> /g)	Mesopores volume (cm <sup>3</sup> /g)	Mean diameter (Å)	d <sub>100</sub> (Å)	a (Å)	Wall thickness (Å)
NH <sub>2</sub> -MSN	789	1.18	30	38.21	44.12	14
HA200A	494	0.91	26	37.88	43.74	18
HA200B	204	0.33	<20	36.32	41.94	>22
HA6.4B	222	0.60	≈22	36.78	42.47	20

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**Table 2:** Quantitative analysis, ~~and~~ ζ potential and mean hydrodynamic diameter

Samples	HA loading (wt%)		ζ potential (mV)		Mean hydrodynamic diameter (nm)	
	TGA	Carbazole	water	PBS <sup>a</sup>	water	PBS <sup>a</sup>
NH <sub>2</sub> -MSN	-	-	+35.0 ± 0.9	+12.4 ± 0.7	360.7 ± 11.2	409.3 ± 23.0
HA200A	6.6	8.1	+14.3 ± 1.3	-16.5 ± 0.8	292.7 ± 10.6	309.0 ± 18.2
HA200B	17.4	19.9	-19.8 ± 1.9	-15.7 ± 0.9	253.1 ± 10.5	265.9 ± 20
HA6.4A	n. d.	3.1	+34.5 ± 0.1	-1.3 ± 0.5	313.8 ± 8.4	389.2 ± 13.0
HA6.4B	19.0	18.9	-8.8 ± 0.7	-16.8 ± 0.8	301.9 ± 12.1	354.7 ± 16.1

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n. d.: not determined; <sup>a</sup> 0.1 M, pH = 7.4

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**Table 3.** Main infrared assignments

High frequency		Low frequency		2
Position	Assignment	Position	Assignment	3
3370 cm <sup>-1</sup>	v asym NH <sub>2</sub>	1650 cm <sup>-1</sup>	vC=O (Amide I)	5
3300 cm <sup>-1</sup>	v sym NH <sub>2</sub>	1595 cm <sup>-1</sup>	δ NH <sub>2</sub> (aminoproyl)	6
2930 cm <sup>-1</sup>	v asym CH <sub>2</sub>	1550 cm <sup>-1</sup>	δNH/vC-N comb. (Amide II) <sup>7</sup>	7
2870 cm <sup>-1</sup>	v sym CH <sub>2</sub>	1430-1350 cm <sup>-1</sup>	δCH and δOH	8

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**Table 4:** Langmuir parameters for water vapour sorption process

Samples	1 <sup>st</sup> cycle			2 <sup>nd</sup> cycle		
	q <sub>m</sub> (wt%)	K	R <sup>2</sup>	q <sub>m</sub> (wt%)	K	R <sup>2</sup>
NH <sub>2</sub> -MSN <sup>a</sup>	6.83	8.47	0.9267	9.23	8.47	0.9267
HA200A	1.37	17.10	0.9932	1.47	18.29	0.9940
HA200B	1.28	20.14	0.9964	1.43	19.47	0.9964
HA6.4B	1.56	20.87	0.9950	1.66	22.82	0.9968

<sup>a</sup> Parameters calculated from data measured at 25 °C (from Ref. [28])

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