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Research paper

Selective internalization of ZnAl-HTlc nanoparticles in normal and tumor cells. A study of their potential use in cellular delivery

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ABSTRACT

A colloidal dispersion of zinc aluminum hydrotalcite nanoparticles (ZnAl-HTlc) has been used for in vitro experimental procedures in order to provide reliable data on their potential application in cellular delivery. Two different cell lines (HeLa tumor cells and MDCK normal cells) with a similar epithelial derivation have been used. Sedimentation studies performed in the presence of different constituents of the cell culture medium revealed the importance of serum components to stabilize the colloidal dispersions of nanosized ZnAl-HTlc. Cell viability assay showed for nanosized ZnAl-HTlc a higher cell growth inhibition on tumor cells compared to normal cells whereas LDH test showed the absence of toxicity for both cell lines. Cellular uptake experiments indicated a preferential internalization of ZnAl-HTlc nanoparticles in HeLa tumor cells. Adsorption study and steady state fluorescence measurements on the phenol red/HTlc hybrid were carried out in order to verify the possibility of using phenol red as fluorescent dye for ZnAl-HTlc nanoparticles. The observed spectral behavior indicated a strong interaction between the dye and the inorganic matrix and the preferential adsorption of the dye on the nanoparticle surface has been confirmed by the XRPD data. Fluorescence confocal imaging showed a different localization pattern of nanosized HTlc in the two cell lines and a higher fluorescence signals in tumor cells supporting the occurrence of more efficient internalization processes in the pathogen cell line as observed in the cellular uptake experiments.

1. Introduction

The field of nanomaterials is a subject of rapid growing interest because it has been well established that materials at the nanoscale exhibit physical, chemical, electrical and biological properties, significantly different from their conventional formulations. These specific characteristics are opening new opportunities for producing materials with surprisingly unusual properties that allow applications in different fields such as catalysis, agriculture, electronics, and biotechnology including cosmetics, pharmaceutics, and medicines (Konstantatos and Sargent, 2010; Lim and Lee, 2010; Sanguansri and Augustin, 2006).

Nanomedicine formulations are frequently used to improve the therapeutic value of various water soluble/insoluble medicinal drugs and bioactive molecules by improving bioavailability, solubility and retention time (Wagner et al., 2006; Zhang et al., 2008). Recently, some researchers have focused on the use of nanoparticles as effective non-viral agent for cellular and gene delivery and on the influence in these fields of their physico chemical characteristics (De et al., 2008; Ghosh et al., 2008; Xu et al., 2006).

The size distribution of nanoparticles is important to determine their interaction with the cell membrane and their penetration across the physiological drug barriers (Fadel and Garcia-Bennet, 2010). The nanoparticle surface composition would determine whether the nanoparticles would cluster in blood flow or adhere to surface-active molecules and whether the resulting nanostructured biomaterial would interact with cell membranes for cellular uptake (Thanh and Green, 2010). Positive surface charge is desirable as it promotes interaction of the nanoparticles with the cells and hence increases the rate and extent of cellular uptake.

Hydrotalcite like-compounds (HTlc), also known as anionic clays, are a particular type of lamellar solids with cationic brucite-like layers and charge-balancing anions in the interlayer region. (Jones and Newman, 1998; Khan and O'Hare, 2002). Synthetic HTlc have general formula $[M(II)_{1-x}M(III)_x(OH)_2]^{x+}[A^{n-}{}_{x/n}]^{x-}.mS$, where M(II) is a divalent cation (typically Mg, Zn, Ni, Co), M(III) is a trivalent metal cation (Al, Fe, Cr), x is M(III)/M(III) + M(II) molar ratio that ranges between 0.25 and 0.4, A^{n-} is an inorganic or organic anion of charge n^-, and m are the mol of solvent S (generally water) intercalated per

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formula weight of compound (Leroux and Taviot-Guého, 2005; Rives, 2001; Trifirò and Vaccari, 1996).

In recent years, HTlc with different metal cations in the layers and consequently diverse exchangeable characteristics have been used in several fields as anion exchangers, catalysts and catalyst supports, polymeric fillers and biosensors (Costantino et al., 2007, 2009; Latterini et al., 2007a, 2007b; Mousty et al., 2008; Turco et al., 2004). Their cationic layered framework leads to safe accommodation of many biologically important molecular anions including genetical material or drugs (Choi et al., 2008; Choy et al., 2000; Costantino et al., 2008; Del Hoyo, 2007; Ladewig et al., 2010a, 2010b; Oh et al., 2006a, 2006b; Pan et al., 2010; Perioli et al., 2008; Qin et al., 2010; Ricci et al., 2005).

Recently, zinc aluminum hydrotalcite nanoparticles have been prepared and characterized (Bellezza et al., 2009a) and used as drug delivery systems of diclofenac (Perioli et al., 2011).

Owing to their high flexibility in compositions, HTlc can be prepared with many types of metal cations and interlayer anions. For applications as health care products HTlc containing Mg(II) and Al(III) are usually preferred. An investigation on the potential use in these fields of different type of hydrotalcite like ZnAl-HTlc could be useful to increase the number of available compounds. However, their biocompatibility and their toxicity need to be tested.

For in vitro studies, pH, ionic strength and the presence of proteins or other surface-active molecules in the medium might have an impact on nanoparticles dispersion behavior. Many studies were reported on micro-crystals, but not so many data are present in the literature for nano-sized ZnAl-HTlc. Instead it was reported that the particle dimension/morphology has to be taken into account to classify their physico-chemical behavior (Stone et al., 2010).

For effectively tracing the pathway of HTlc nanoparticles in the cells, HTlc functionalization with a stable, non-invasive and highly sensitive fluorescent label is necessary (Musumeci et al., 2010). Generally, in-vitro studies were so far conducted using HTlc nanoparticles labeled with fluorescein isothiocyanate (FITC) as fluorescent dye. The FITC labeling of HTlc nanoparticles may be done or by an intercalation process, through an ion exchange mechanism (Xu et al., 2008) or by a covalent bonding realized with silane coupling reaction and thiourea bond formation (Oh et al., 2009). In both cases to achieve detectable optical properties several synthetic steps are necessary. In order to avoid this procedure an alternative labeling method which makes use of a component of the cell culture medium will be described in this paper.

Usually the synthetic cell culture medium contains phenol red (also known as phenolsulfonphthalein or PSP) as pH indicator, which turns from yellow to intense pink color in the pH range 6.4–8.2 since it has a pKa value of 8.0. However it has recently reported (Srour and McDonald, 2008) that its pKa value decreases of more than one unit in medium with dielectric constant lower than water; therefore, at physiological pH values (pH = 7.4) it is mainly in basic form thus bearing a negative charge. The electrostatic repulsions between the negatively charged cell membrane and the dye molecules are likely responsible for the lack of an efficient uptake of the indicator molecules by the cells. Only in highly sensitive detection setups for advanced fluorescence microscopy techniques PSP fluorescence appears as diffused fluorescence light which, in these conditions, might interfere with the measurements (Stephens and Allan, 2003).

On the other hand, the basic form of PSP could adsorb via electrostatic interactions onto the positively charged brucite-like layers functioning as a fluorescent label for HTlc. The interactions between PSP and HTlc lead to the formation of a fluorescent composite material with reduced net charge, which could facilitate the PSP transfer into the cells.

In this study we investigated the cellular uptake of ZnAl-HTlc nanoparticles and the possibility of using PSP as fluorescent dye. Furthermore, a detailed study on the stability of ZnAl-HTlc nanoparticles in cell culture medium and on their relative toxicity is reported. Two different cell lines, tumor (HeLa) and normal cells (MDCK) of the same derivation were employed as they are widely used as a representative model of epithelial tissue. Their common embryonal origin allows us to have a more representative comparison of the ZnAl-HTlc effects on tumor and normal immortalized cells.

2. Experimental section

2.1. Preparation of HTlc nanoparticles

ZnAl-HTlc was obtained as previously described (Bellezza et al., 2009a). Briefly, two microemulsions designated A and B were prepared by dispersing 12.5 g (0.034 mol) of cetyltrimethylammonium bromide (CTABr), 15.5 mL (0.169 mol) of n-butanol, 36.2 mL (0.219 mol) of isooctane and 13.5 mL of aqueous phase. The aqueous phase of A was a solution of $Zn(NO_3)_2 \cdot 6H_2O$ (0.4 M) and $Al(NO_3)_3 \cdot 9H_2O$ (0.125 M), while the aqueous phase of B was a NH_3 solution (1.25 M). Equal volumes of A and B microemulsions were mixed to obtain the precipitation of ZnAl-HTlc in the reverse micelles. The resulting system was aged at 75 °C for 15 h. After aging, the particles were recovered by centrifuging (12,000 rpm for 10 min). The precipitate was washed with ethanol–chloroform mixture (1:1 v/v) (3 × 30 mL) and then with water (3 × 30 mL).

Finally, it was dispersed in water to have a colloidal dispersion which was characterized in terms of nanoparticles concentration by a gravimetric method.

2.2. Characterization of ZnAl-HTlc

The X-ray powder diffraction (XRPD) patterns were taken with a Philips X'PERT PRO MPD diffractometer operating at 40 kV and 40 mA, step size 0.0170 2 Theta degree and step scan 20 s, using the CuKα radiation and an X'Celerator detector.

Zeta potential measurements were performed by a Nicomp 380 ZLS (PSS, CA), equipped with a HeNe Laser source at 632.8 nm by applying 1 V/cm electric potential and measuring the scattered light at 14.9°.

The morphology and the particle size were investigated with a Jeol 2010 Transmission Electron Microscope (TEM), operating at 200 kV. A small drop of the aqueous dispersion was deposited on a copper grid pre-coated with a Formvar film and then evaporated in air at room temperature.

2.3. Sedimentation studies in different aqueous media and in cell culture media

The ZnAl-HTlc dispersions in water were sonicated for 10 min and then were added to different aqueous media in order to achieve the final target concentration of 250 μg/mL for ZnAlHTlc nanoparticles. The sedimentation process was monitored with a UV–vis spectrophotometer by following the absorbance at 600 nm every 30 s for 6000 s. The measurements were carried out at 20 °C.

2.4. Cell culture

Madin–Darby canine kidney cells (MDCK) and human cervical adenocarcinoma epithelial cells (HeLa) were cultured in Dulbecco's modified eagle's medium (DMEM) in humidified atmosphere (5% CO_2) at 37 °C. All media were supplemented with 10% heat inactivated fetal bovine serum (FBS) (Euroclone), 100 units/mL penicillin, and 100 μg/mL streptomycin. For the reader convenience the concentrations of some DMEM and FBS components were listed in Table 1.