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Platinum folate nanoparticles toxicity: Cancer vs. normal cells

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ABSTRACT

Almost for two decades metallic nanoparticles are successfully used for cancer detection, imaging and treatment. Due to their high electron density they can be easily observed by electron microscopy and used in laser and radiofrequency therapy as energy releasing agents. However, the limitation for this practice is an inability to generate tumor-specific heating in a minimally invasive manner to the healthy tissue. To overcome this restraint we proposed to use folic acid coated metallic nanoparticles and determine whether they preferentially penetrate cancer cells.

We developed technique for synthesizing platinum nanoparticles using folic acid as stabilizing agent which produced particles of relatively narrow size distribution, having $d = 2.3 \pm 0.5$ nm. High resolution TEM and zeta potential analysis indicated that the particles produced by this method had a high degree of crystalline order with no amorphous outer shell and a high degree of colloidal stability. The keratinocytes and mammary breast cells (cancer and normal) were incubated with platinum folate nanoparticles, and the results showed that the IC50 was significantly higher for the normal cells than the cancer cells in both cases, indicating that these nanoparticles preferentially target the cancer cells. TEM images of thin sections taken from the two types of cells indicated that the number of vacuoles and morphology changes after incubation with nanoparticles was also larger for the cancer cells in both types of tissue studied. No preferential toxicity was observed when folic acid receptors were saturated with free folic acid prior to exposure to nanoparticles. These results confirm our hypothesis regarding the preferential penetration of folic acid coated nanoparticles to cancer cells due to receptor mediated endocytosis.

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1. Introduction

Metallic nanoparticles such as gold, silver and platinum were successfully used for cancer detection, imaging and treatment for more than a decade (Arvizo et al., 2012; Bellah et al., 2012; Govender et al., 2012; Reddy et al., 2012; Veerasamy et al., 2011; Wang et al., 2012). Due to their high electron density they can be easily observed by electron microscopy and can be used in laser and radiofrequency therapy as energy releasing agents (Elliott et al., 2010; Elsherbini et al., 2011). This type of treatment induces coagulative necrosis to the cell by protein thermal denaturation and membrane lysis. However, inability to generate tumor-specific heating in a minimally invasive manner to the healthy tissue lead to the usage of single point source of thermal energy that is not uniform along the tumor resulting in uneven heating and tumor recurrences.

To overcome this limitation metallic nanoparticles have to preferentially penetrate cancer cells. Even though numerous targeting strategies have been proposed (Gao et al., 2012; Glazer and Curley, 2010; Sanna and Sechi, 2012; Yang et al., 2012), most are fairly complicated, relying on the isolation of specific cell membrane receptors or their conjugates. This renders them not only costly, but useful only to one specific cell line. Here we present an alternative approach which is simple, relatively inexpensive, and promising.

It has been reported by numerous groups (Basal et al., 2009; Kalli et al., 2008; Kularatne and Low, 2010; Liang et al., 2011; Low et al., 2008; Salazar and Ratnam, 2007; Tran et al., 2005) that cancer cells have significant up regulation of folic acid receptors. There are three folate receptor (FR) isoforms (FR-α, FR-β and FR-γ) that have been identified in human tissues and tumors. FR-α and FR-β are known to be vastly over expressed in many human tumors, unlike normal tissues expressing insignificant levels of FR-α and low levels of FR-β (Ross et al., 1994; Vaitilingam et al., 2012). FR-γ is only found in hematopoietic cells. Consequently, folate has been widely used as a targeting moiety of various anticancer drug systems (Chen et al., 2011; Huang et al., 2011; Ji et al., 2012; Low and Antony, 2004; Lu et al., 2012; Mishra et al., 2011; Werner et al., 2011). Since, it has been

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shown by numerous authors that folate-drug conjugates can quickly bind to the FR receptors and deliver their payload (Vlahov and Leamon, 2012; Xu et al., 2012). The FR-mediated drug delivery has been referred to as a molecular Trojan horse approach where drugs attached to folate are shuttled inside targeted-FR-positive cells in a stealth-like fashion.

Despite the efficacy, folate is rarely used to coat metallic particles. Even though the synthesis can be accomplished in a simple two steps process, little is known about the parameters controlling the particle size. For example, the only other reported synthesis yielded 15 nm nanoparticles at room temperature (Teow and Valiyaveettil, 2010). Since it is well established (Freese et al., 2012; Mironava et al., 2010; Pan et al., 2007; Zhang et al., 2012) that the interaction of NP with cell is a sensitive function of particle diameter, the particles synthesized in this paper, which are 2 nm in diameter are expected to interact with cells quite differently than 15 nm particles. Furthermore, in order to employ these particles for therapeutic purposes, it is also critical to establish the differential toxicity between normal and cancer cells.

In this paper we expose both cancer and normal cells of the same tissue to the folate stabilized platinum nanoparticle (Pt/folate NPs) and demonstrate that they are preferentially internalized in the cancer cells, where they have a significantly smaller IC50 level. Hence they can potentially be used for anti-cancer drug conjugation to be tested in cancer treatment protocols.

2. Materials and methods

2.1. Synthesis of Pt/folate nanoparticles

Pt/folate nanoparticles were synthesized utilizing 0.005 mol of the oxidant, potassium tetrachloroplatinate(II) (K_2PtCl_4) and 0.005 mol of the stabilizer, folic acid ($C_{19}H_{19}N_7O_6$) in 20 ml MilliQ deionized water. The Erlenmeyer flask containing this solution was heated up to 95 °C for about 20 min to fully dissolve potassium tetrachloroplatinate and folic acid. Over a couple of minutes the color of solution changed from yellow to brownish and after that the solution was allowed to naturally cool down. 20 μl of 0.019 M of sodium tetrahydridoborate ($NaBH_4$) was gradually added up to the cooled solution, until solution color changed from brownish to black indicating reaction completion. The reaction differs from the previously reported by Teow et al. where alternative precursor was used and the synthesis was performed at room temperature (Teow and Valiyaveettil, 2010).

2.2. Cell culture

Primary human epidermal keratinocytes were obtained from Stony Brook University Living Skin Bank. Normal Keratinocytes DO33 and two phenotypically distinct squamous carcinoma keratinocytes SCC13 and SCC12B were grown in the keratinocytes growth media KGM-2 supplemented with bullet kit (Cat# CC-3107, Lonza, Allendale, NJ) containing 0.4% bovine pituitary extract, 0.1% human recombinant epidermal growth factor, 0.1% insulin, 0.1% hydrocortisone, 0.1% transferrin, 0.1% epinephrine and 0.1% gentamicin sulfate amphotericin-B. SCC12 and SCC13 were initially isolated by Rheinwald and Beckett (1981). The cells used were derived from these lines through growth and passage in culture.

MCF7 – mammary breast cancer cells, 69 years-old Caucasian female (Cat# HTB-22, ATCC, Manassas, VA) were cultured with ATCC-formulated Eagles' Minimum Essential Medium (Cat# 30-2003, ATCC, Manassas, VA) with 0.01 mg/ml insulin and 10% of fetal bovine serum (Lonza, Allendale, NJ).

MCF10A – mammary breast cells, 36 year-old Caucasian female (Cat# CRL-10317, ATCC, Manassas, VA) cells were cultured with Lonza single kit medium containing 2.5 mM L-glutamine and supplemented with 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone, 95% and 5% horse serum (Lonza, Allendale, NJ).

Cells were plated at cell density 35,000 cells per well in a 96-well dish for MTS assay or in a 24-well dish for the confocal microscopy respectively. Cells were treated with following concentrations of platinum/folate nanoparticles 0 μg/ml, 25 μg/ml, 50 μg/ml, 75 μg/ml, 100 μg/ml, 125 μg/ml,150 μg/ml, 175 μg/ml, 200 μg/ml, 225 μg/ml, 250 μg/ml and 300 μg/ml 24 h after platting. Samples were collected at specific time points (12 and 24 h) and were MTS assayed. All incubations were performed at 37 °C and 5% CO_2. Following doubling times were determined for the cells used in this study: MCF7 – 29 h, MCF10A – 34 h, DO33 – 32 h, SCC13 – 18 h, SCC12B – 17 h.

2.3. MTS

Cell mitochondrial activity was evaluated with CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Biosciences, Madison, WI). In the typical experiment cells were platted at the initial density 35,000 cells per well in 96-well dishes. 20 μl of the MTS solution was added to the 100 μl of media, samples were incubated for 3 h at 37 °C. The absorbance was read at 490 nm by the automated microplate reader EL×800.

2.4. Cell staining for confocal microscopy

Cell area and overall morphology as a function of time and concentration was monitored using Leica confocal microscope. For these experiments, cells grown on cover slip glass in 24-wells dishes were fixed with 3.7% formaldehyde for 15 min following exposure to Pt/folate NPs for 12 and 24 h. Alexa Fluor 488–Phalloidin was used for actin fiber staining and Propidium Iodide for nuclei staining.

2.5. TEM

TEM analysis was used to assess the size distribution of the Pt/folate NPs as well as the fate of internalized particles. One drop of the original Pt/folate NPs solution was placed on 300 mesh copper grip, which was coated with formvar film. The sample was then dried out at room temperature. Gaussian distributions of diameters were calculated from the samples with more than 170 nanoparticles. After exposure to Pt/folate NPs for 2 days the cells were fixed in a solution of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M Phosphate Buffered Saline (PBS), stained in 2% uranyl acetate, dehydrated with ethanol, and then embedded in Propylene oxide. The specimen was cut into ultrathin sections (90 nm) with Reichart UltracutE ultramicrotome and stained on the grid with uranyl acetate and lead citrate. The samples were imaged using a FEI Tecnai12 BioTwinG2 transmission electron microscope. Digital images were acquired with an AMT XR-60 CCD Digital Camera System and compiled using Adobe Photoshop program.

2.6. pH

pH-meter calibrated with standard buffers was used to measure pH value of the freshly prepared Pt/folate NPs colloidal suspensions. pH electrode was immersed in the NPs suspension for 30 s or until pH value stabilized. All measurements were performed in triplicates at 25 °C.