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ORIGINAL CONTRIBUTION

Self-assembling chitosan/poly-γ-glutamic acid nanoparticles for targeted drug delivery

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Abstract For the purpose of targeted drug delivery, composite biodegradable nanoparticles were prepared from chitosan and the poly-γ-glutamic acid via an ionotropic gelation process. These stable self-assembled nanoparticles were characterized by dynamic light scattering, transmission electron microscopy, and atomic force microscopy, which demonstrated that the nanosystem consists of spherical particles with a smooth surface both in aqueous environment and in dried state. Toxicity measurements showed that the composition is nontoxic when tested either on cell cultures or in animal feeding experiments. To evaluate the potential of the nanosystem for intracellular drug delivery, the nanoparticles were fluorescently labeled and folic acid was attached as a cancer cell-specific targeting moiety. The ability of the particles to be internalized was tested using confocal microscopic imaging on cultured A2780/AD ovarian cancer cells, which overexpress folate receptors. The quantitative data obtained by digital processing of the intensity of green color of each pixel in the pictures inside the cell boundaries and total intensity of fluorescence inside the cells showed that "targeted" particles internalized into the cells significantly faster and the total accumulation of these particles was substantially higher in the cancer cells when compared with "nontargeted" particles, which may facilitate effective and specific cytoplasmic delivery of anticancer agents loaded into such nanoparticles.

Keywords Chitosan · Poly-γ-glutamic acid · PGA · Nanoparticles · Cytotoxicity · *In vivo* toxicity · Cellular uptake

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Introduction

A variety of nanoscale drug delivery systems have been investigated over the past decades to supplement conventional systemic chemotherapy in order to enhance the efficacy of traditional anticancer drugs and prevent their adverse side effects. Polymeric nanoparticles (NPs) among other systems such as drug–polymer conjugates, drug-loaded liposomes, polymeric micelles, and polymersomes are promising solutions to the problem as within such formulations cytotoxic drug molecules are encapsulated and their release can be concentrated at tumor sites [1–4]. NPs may prove superior owing to their high drug loading capacity, controllable size and permeability, and protection of the drug from metabolism or early clearance. Preferred features for such particles with respect to chemistry are biocompatibility, nontoxicity, nonimmunogenicity, and biodegradability, which allow repeated systemic administration

of the formulation and ultimate clearance of the particles from the body [5]. However, ideal drug-loaded carriers have to be complex multifunctional entities, e.g., to achieve a high therapeutic index, (a) they should remain in circulation for prolonged time by escaping the body's natural filtration systems (e.g., reticuloendothelial system), (b) accumulate specifically or nonspecifically at the targeted pathological zone, (c) be responsive to local conditions (e.g., low pH or elevated temperature at neoplastic tissues) so as to liberate the cargo, and (d) to promote intracellular–subcellular delivery of the drug for efficient tumor cell killing [6–8].

Although drug-loaded NPs can passively accumulate in tumors as a result of the enhanced permeability and retention effect caused by the tumor-associated leaky vasculature, efficient tumor control requires active targeting of the delivery vehicle via specific and differential interaction with surface proteins/receptors on cancer cells. Antibodies and their fragments against tumor-specific antigens, peptide ligands, hormones (e.g., luteinizing hormone-releasing hormone), various proteins (e.g., transferrin), lectins, sugars, or low molecular weight compounds (e.g., folate) have been successfully used as targeting moieties of therapeutic NPs [2–4, 6].

Polymer research into suitable chemical compositions for "smart" nanovehicles, which entrap, solubilize, localize and control drug release, is very intense, and aims to develop complex structures of high molecular weight, defined three-dimensional structure and tailored surface valency. Typical synthetic polymer solutions designed exclusively for this purpose include dendrimers, dendronized polymers, hyperbranched, block copolymers, and hybrid glyco and peptide derivatives [2–4, 9, 10]. Although they are nonnatural polymers, dendrimers (tree-like polymers) have attracted attention because they have a well-defined structure, a monodisperse colloidal nature, and they offer flexibility in terms size, shape, branching, length, and surface functionality [2]. Still, natural biodegradable polymer-based NPs have several advantages over the nondegradable counterparts such as the inherent possibility to control drug release through biodegradation of the polymer composition within cells [5].

Herein, we report the preparation of self-assembling composite polymeric nanoparticles from the natural polymers, the polycationic chitosan (CS) and polyanionic poly-γ-glutamic acid (γ-PGA) via an ionotropic gelation process [11–15]. Chitosan is a renewable basic linear biomaterial, containing β-[1→4]-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. It is a nontoxic, biocompatible, and biodegradable polysaccharide with low immunogenicity and antibacterial properties. γ-PGA is a water soluble, biodegradable, edible, and nontoxic polyanion for the environment and humans [13, 16].

In our previous work [11], we discussed the successful preparation and characterization of nanocomplexes based on self-assembly of poly-γ-glutamic acid and chitosan. It was described the effect of reaction conditions on formation of nanosystems. Solubility, surface charge, size, and size distribution of these nanosystems were studied depending on the concentration and ratio of biopolymers, pH environment as well as order of addition. In light of potential use of self-assembled nanoparticles as targeted drug delivery vehicle, we chose characterized a special nanosystem from them for cytotoxicity and *in vivo* toxicity on cultured cells and animal experiments and demonstrate the preferential uptake by cancer cells of the nanoparticles when they are conjugated with a tumor-specific targeting moiety.

Experimental section

Materials

Chitosan (degree of deacetylation 88%, M_v=3.2×10^5) was purchased from Sigma-Aldrich, Co., Hungary. CS was purified as follows: CS was dissolved in 2.0% aqueous acetic acid solution to give a polymer concentration of 1.0% *w/w* and then filtered and dialyzed against distilled water until the pH was neutral. The product was dried by lyophilization to obtain a white chitosan powder and used for further experiments. Poly-γ-glutamic acid (M_W=1.2×10^6, GPC) was prepared in our laboratory by using the biosynthetic methods described earlier [17, 18]. Water-soluble 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide methiodide (EDC) and folic acid dihydrate (FA) were purchased from Sigma-Aldrich, Co., Hungary.

Formation

Synthesis of folated γ-PGA (γ-PGA-FA) FA was conjugated (via the amino group of pteridine moiety) to poly-γ-glutamic acid using water soluble carbodiimide (EDC). After the dropwise addition of EDC (8 mg in distilled water) to the γ-PGA solution (50 ml, 1 mg/ml, pH 6.5), the reaction mixture was stirred at room temperature for 30 min. Folic acid (12 mg in dimethyl sulfoxide (DMSO)) was added and stirred at room temperature for 24 h. The γ-PGA-FA conjugate was purified by dialysis and the number of FA molecules per γ-PGA was estimated by UV–VIS absorption spectroscopy (λ_{max1} 368 nm, ε 9,120; λ_{max2} 283 nm, ε 25,100) to eventuate in an average of seven FA molecules attached to one PGA molecule.

Synthesis of fluorescein-labeled chitosan Chitosan solution (10 ml, 1 mg/ml in water, solubilized with HCl and pH adjusted to 6.5 with NaOH) was mixed with an aliquot of fluorescein

isothiocyanate (FITC; 1 mg/ml in DMSO, 250 μl) and the reaction mixture was stirred at room temperature for 24 h [14, 15]. Fluorescein-labeled chitosan (CS-FITC) was purified by dialysis against water (3 days) and characterized by UV–VIS spectrophotometry and an average value of 71 fluorescein moiety per chitosan molecules was observed.

Preparation of CS-FITC/γ-PGA-FA nanoparticles Stable self-assembled polyelectrolytes were developed via an ionotropic gelation process between the folated γ-PGA and the fluorescently labeled chitosan linear chains. When an equal volume of aqueous γ-PGA-FA (0.3 mg/ml, pH 9.0) was added (1 ml/5 s) into CS-FITC (0.3 mg/ml, pH 4.0) solution under continuous stirring, an opaque colloidal system was formed (75% transmittance at λ 500 nm, pH 7.4), which remained stable at room temperature for several weeks at physiological pH.

Characterization

Transmission electron microscopy and atomic force microscopy A JEOL2000 FX-II transmission electron microscope was used to characterize the size and morphology of the dried nanoparticles as described before [11, 19, 20]. The samples for transmission electron microscopy (TEM) analysis were obtained by placing a drop of the colloid dispersion containing the nanoparticles onto a carbon-coated copper grid. It was dried at room temperature. Mean diameters and the size distributions were obtained using the SPSS 11.0 program. Samples for atomic force microscopy (AFM) were prepared by casting a drop of the nanoparticle suspension (0.1 mg/ml) on a glass slide; then, it was dried under vacuum. Nanoparticles were analyzed on a WITec alpha300 A/R equipment using the tapping mode.

Laser light scattering Hydrodynamic diameters of nanoparticles (0.1–1 mg/ml) were gauged by using a BI-200SM Brookhaven Research laser light scattering photometer equipped with a Nd/YAG solid-state laser at an operating wavelength of λ_0=532 nm. Measurements of the average size of nanoparticles were performed at 25 °C with an angle detection of 90° in optically homogeneous quartz cylinder cuvettes. Each sample was measured in triplicates, and average serial data were calculated.

Electrokinetic measurement Electrokinetic mobility of the nanoparticle was measured at 25 °C with a Malvern Nanosizer Nano-ZS apparatus. Sample was measured three times and average serial data were calculated.

Cell line The human multidrug resistant ovarian carcinoma A2780/AD cell line was obtained from Dr. T. C. Hamilton (Fox Chase Cancer Center). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Fisher Chemicals, Fairlawn, NJ, USA). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2 (*v*/*v*) in air. All experiments were performed on cells in the exponential growth phase.

In vitro cytotoxicity Experiments were carried out on A2780 human ovarian cancer cells that overexpress folate receptor. The cytotoxicity of nanoparticles was assessed using a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [21, 22]. Briefly, cells were seeded into 96-well microtiter plates at the density of 10,000 cells per well. Twenty-four hours after plating, medium was aspirated and 12 various concentrations (from 0.3 mg/ml to 147 ng/ml) of nanoparticles in fresh media were separately added. Control cells were treated by an equivalent volume of fresh media. Cells were cultured for 24 h with nanoparticles before the cell survival assay was performed. The medium was discarded and 100 μl of fresh medium and 25 μl of a 5-mg/ml MTT solution in Dulbecco's phosphate buffered saline was added to each well. Plates were incubated under cell culture conditions for 3 h. Formazan crystals were dissolved overnight in 50% (*v*/*v*) dimethylformamide in water containing 20% (*w*/*v*) sodium dodecyl sulfate. The absorbance of each sample was measured at 570 nm with a background correction at 630 nm. A modified MTT assay is being routinely used in our laboratories to measure cytotoxicity of different drugs in cell culture experiments. This method allows testing drugs in a wide range of concentrations (2^{10}–2^{44}) with acceptable reproducibility and quantitative analysis using conventional microtiter plate reader. The method of calculation and corresponding computer program were developed and extensively tested [23–26].

In vivo toxicity Experiments were carried out on three groups of nude mice (four animals per group). After systemic injection of saline or two types of nanoparticles, mice body weight was measured everyday within 1 week after the injection. The decrease in body weight on 10% or more when compared with control animals (injection of saline) is usually considered as a criterion of toxicity [27].

Intracellular localization of nanoparticles To analyze intracellular localization of nanoparticles, a portion of chitosan nanoparticles with or without folate were labeled by FITC. Intracellular localization of nanoparticles was studied by confocal microscopy. The internalization was examined by confocal microscopy in living cells at 37 °C within 1 h as previously described [28].

Statistics All *in vitro* and *in vivo* experiments were performed in quadruplicate. The results are expressed as mean ± standard deviation (SD) from four independent

measurements. Statistical analysis was performed as a one-way analysis of variance and comparisons among groups were performed by independent sample *t* test.

Results and discussion

Preparation of nanoparticles To assess the suitability of chitosan/poly-γ-glutamic acid nanosystem for drug delivery, first we needed to incorporate additional components to allow cancer cell-specific targeting and detection of cellular uptake. As a targeting moiety, we chose the vitamin FA, which has a high affinity for folate receptors overexpressed in a number of epithelial and myeloid cancer cells [2, 29].

Characterization of nanoparticles The presence of individual nanoparticles was confirmed and their size distribution characterized by TEM, AFM, and dynamic light scattering (DLS) [11]. The analyses demonstrated that the CS-FITC/γ-PGA-FA nanosystem consists of spherical particles with a smooth surface both in aqueous environment and in dried state (Fig. 1a, b). TEM micrograph showed a size range of 30–160 nm with a mean value of 67.8 nm (Fig. 1b), while DLS reported a bimodal distribution for hydrodynamic diameter ranging between 70–90 and 160–200 nm with mean values of 80 and 178 nm, respectively (Fig. 2). The difference in size of nanoparticles in the dried and solvated phase is consistent with

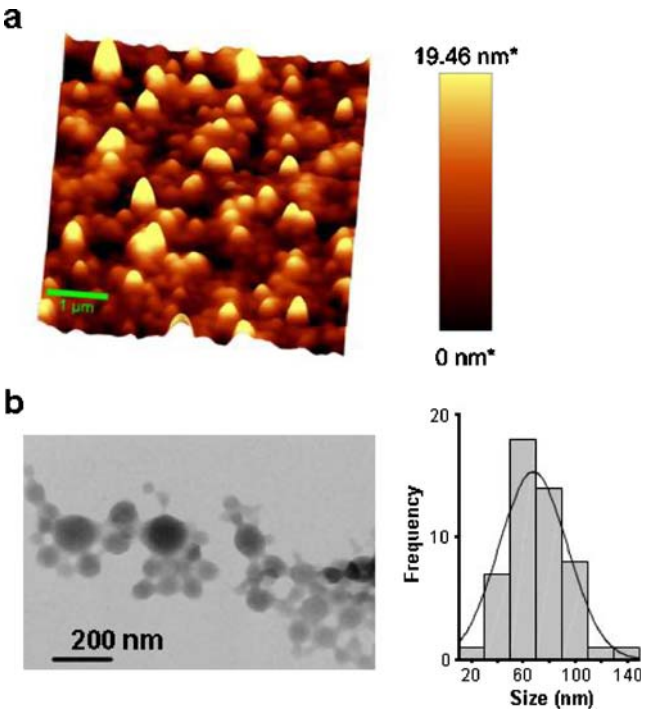

Fig. 1 Characterization of chitosan-FITC/poly-γ-glutamic acid-folate nanoparticles (CS/γ-PGA 1:1, 0.3 mg/ml). **a** AFM micrograph with *color* key for the third dimension, **b** TEM micrograph and particle size distribution

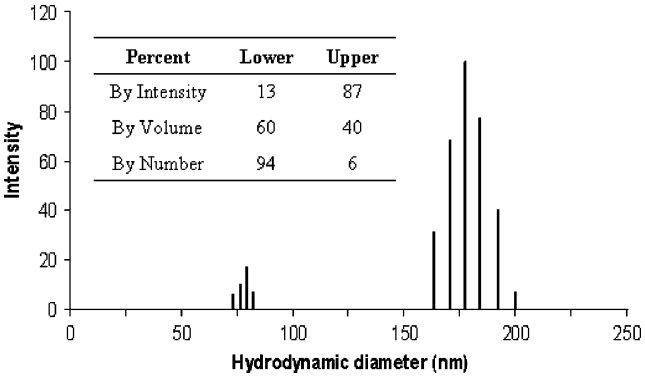

Fig. 2 Size distribution chitosan-FITC/poly-γ-glutamic acid-folate nanoparticles determined by dynamic light scattering

that of a swollen composite biopolymeric system in aqueous media. The bimodal diameter distribution observed by DLS can be explained by interparticle interactions or the statistical distribution of the number of macromolecules (polycations and polyanions) within individual particles.

A prediction for overall charge ratio was performed for the particles. For the estimation, the pKa values of biopolymers were taken in account. Chitosan molecules (pKa≅6.5) [30, 31] in an aqueous solution with pH=4 before the self assemble process practically exist as only polycations. γ-PGA molecules (pKa≅2.2) [30] at pH=9 exist as only polyanions. For the assembled particles, the overall charge ratio (between the positively charge $-NH_3^+$ on CS to the negatively charged $-COO^-$ groups on γ-PGA) of NPs was calculated as +0.67:−1 based on the weight ratio between CS and γ-PGA. This approximation predicts negatively charged nanoparticles. To prove the estimation above, the overall charge ratio of nanoparticles was measured by electrokinetic method. Electrokinetic mobility (u) of CS-FITC/γ-PGA-FA nanosystem was $u=-2.09\pm 0.07$ (m/s)(V/cm). According to our previous results [11] and in agreement with other works, [14, 15] this results in a negative electrokinetic mobility at physiological pH, which may contribute to stabilization of the nanosystem via charge repulsion between individual particles.

Cellular and systemic toxicity of nanoparticles The toxicity of the CS/γ-PGA nanosystem was determined on cultured cells and on whole animals. Two types of fluorescently labeled chitosan/poly-γ-glutamic acid nanoparticles were tested: (a) "nontargeted" control, CS-FITC/γ-PGA (with no folate conjugated to γ-PGA) and the (b) "targeted" CS-FITC/γ-PGA-FA particles (with folate conjugated to γ-PGA). In vitro cytotoxicity of the nanoparticles was assessed on exponentially growing human multidrug resistant ovarian carcinoma A2780/AD cells using a modified MTT assay as described elsewhere [17–22]. Figure 3a shows that no statistically significant differences in cellular viability were found between the experimental and control groups, which

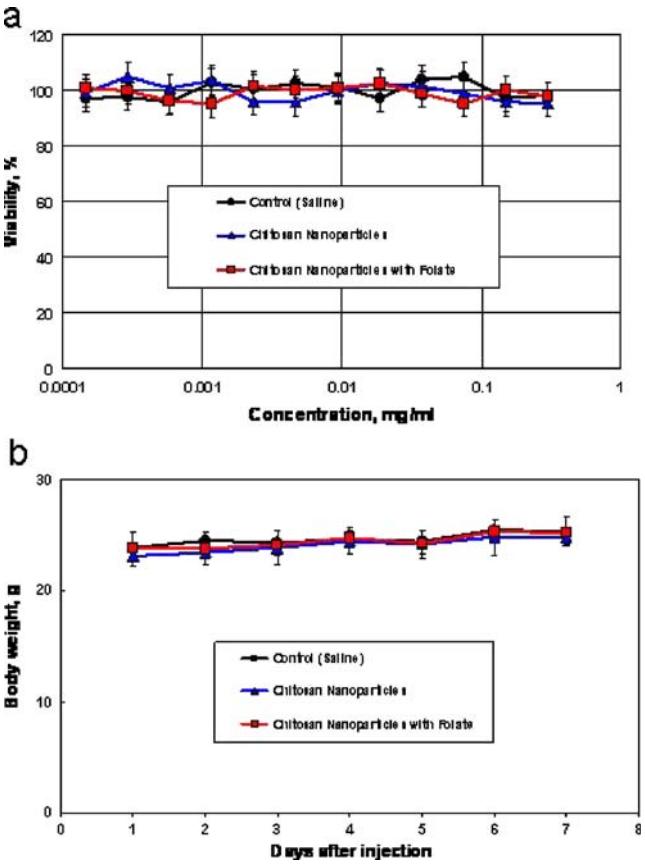

Fig. 3 Toxicity analysis. **a** *In vitro* cytotoxicity of different nanoparticles as measured on A2780 human ovarian carcinoma cells. **b** *In vivo* toxicity as measured via changes in body weight of nude mice after the treatment with different nanoparticles. Means ± SD are shown

excludes cytotoxicity for the CS/γ-PGA composition. In vivo toxicity experiments were carried out on three groups of nude mice (four animals per group). After systemic injection of saline or the two types of nanoparticles, mice body weight was measured everyday within 1 week after the injection. The data presented on Fig. 3b demonstrate that no statistically significant differences in body weight (less than 10%) were found between the experimental and control groups of animals. In summary, the results of the *in vitro* and *in vivo* toxicity measurements clearly showed that both types of nanoparticles (CS-FITC/γ-PGA and CS-FITC/γ-PGA-FA) are nontoxic and therefore, the CS/γ-PGA system can be suitable for systemic administration, e.g., as a drug formulation.

Cellular internalization of nanoparticles The ability of the particles to be internalized was tested on cultured A2780/AD ovarian cancer cells which overexpress folate receptors using confocal microscopy as previously described [28]. To show that nanoparticles with or without folate can penetrate into the cellular cytoplasm, an aliquot was added directly to culture media. Typical transmission and fluorescent images obtained in these experiments (Fig. 4) showed that FITC-labeled particles with folate readily penetrated the cell within 60 min. In contrast, particles lacking folate did not readily penetrate the cell and even after 60 min, there was very limited uptake. Within the 60 min of observation, "targeted" particles (CS-FITC/γ-PGA-FA) filled up the total volume of the cytoplasm while remaining completely excluded from the nucleus. In case of "nontargeted" particles, some fluorescence appeared within the cell cytoplasm; however, the signal was significantly weaker compared to that observed for targeted particles.

To quantitatively estimate the accumulation of FITC-labeled nanoparticles, digital photographs were processed by a program developed in our laboratories. The intensity of green color of each pixel in the picture inside the cell boundaries was measured and total intensity of fluorescence inside the cell was calculated. The results of the calculation are presented in Fig. 5. The quantitative data obtained showed that "targeted" particles internalized into the cells significantly faster and the total accumulation of these particles was substantially higher in the cancer cells when compared with "nontargeted" particles.

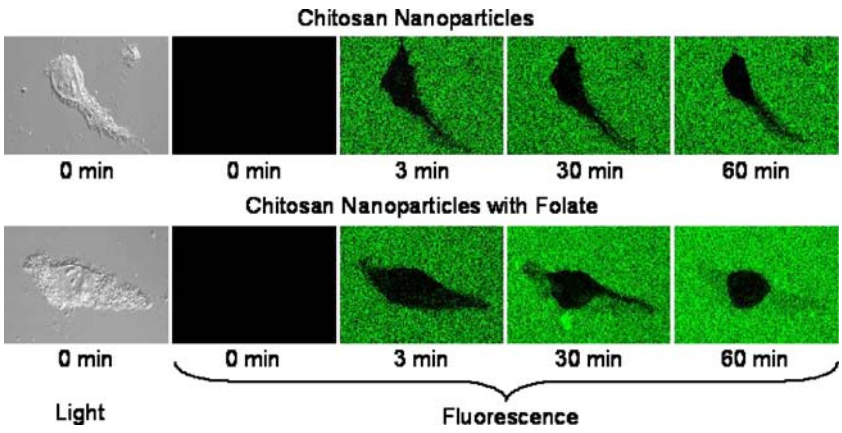

Fig. 4 Confocal microscopy in living A2780 human ovarian cancer cells at 37 °C before and after incubation with FITC-labeled chitosan/γ-PGA nanoparticles with (*upper panel*) and without folate (*lower panel*)

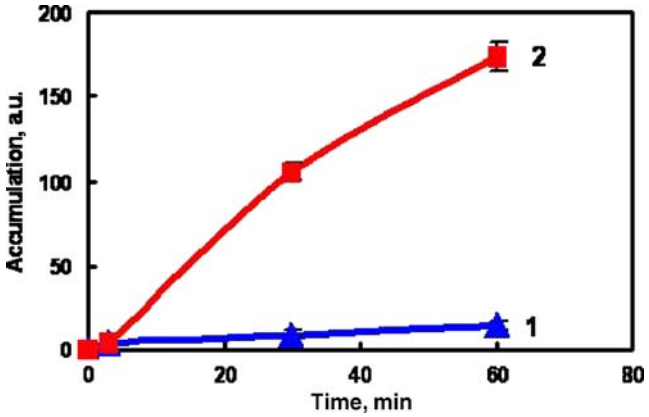

Fig. 5 Accumulation of FITC-labeled chitosan/γ-PGA nanoparticles without (*1*) and with folate (*2*) in A2780 human ovarian cancer cells at 37 °C. The intensity of *green color* of each pixel in confocal images (Fig. 4) inside the cell boundaries was measured and total intensity of fluorescence inside the cell is plotted against time

Theoretically, "targeted" nanoparticles could adhere to the surface of cancer cells and erroneously be visualized on microscopic images as internalized within the cells. To exclude such errors, we analyzed the distribution of labeled microparticles in different cellular layers from the upper to the lower surfaces of the cells using a confocal fluorescent microscope (*z*-sections; Fig. 6). The data obtained show that the distribution of labeled "targeted" nanoparticles in cellular cytoplasm was uniform and very similar in different cell layers. In conclusion, the use of folate as a targeting moiety specific to cancer cells enhances cellular internalization of CS/γ-PGA nanoparticles.

Conclusions

Our results demonstrate the potential of a chitosan/poly-γ-glutamic acid-based self-assembling nanoparticulate system as a drug delivery platform. The two main components of this polycationic-polyanionic gel-type composite nanosystem are renewable as chitosan is derived from chitin of crustacean shell by alkaline deacetylation while γ-PGA is also easily obtained from *Bacillus* sp. ferments, where it is produced as slime. CS and γ-PGA are known to be fully biocompatible, biodegradable, and likely nonimmunogenic and also failed to display any toxicity in our cellular and *in vivo* studies. Also, the two polymers have a wide range of biomedical applications in separate or in combination [12, 13]. Additional advantages of the CS/γ-PGA nanosystem as nanocarrier are that (a) its self-assembling nature provides simple preparation without resorting to chemical cross-linking, organic solvents, or other toxic additives, (b) the use of degraded polymers facilitate particle size control, and that (c) surface charge and functionality of NPs are conveniently tunable by varying component mixing ratios. A similar nanosystem has recently been tested for oral insulin delivery, where insulin complexation and paracellular transport via the epithelium was achieved by NPs assembled from degraded CS and γ-PGA [14, 15]. In this work, we demonstrated that CS/γ-PGA nanoparticles can penetrate A2780/AD ovarian cancer cells and that a significantly faster and more efficient transport is observed in the presence of conjugated folic acid. This may facilitate effective cytoplasmic delivery of anticancer agents loaded into such nanoparticles via either covalent attachment or noncovalent association to charged components or to introduced hydrophobic moieties [32]. All the above features make this CS/γ-PGA NP system an excellent scaffold for building multifunctional nanocarriers for targeted drug delivery or related medical applications such as diagnostic imaging and drug screening. Further studies aiming detailed molecular characterization of the nanoparticles and the mechanism and specificity of their cellular uptake are under way.

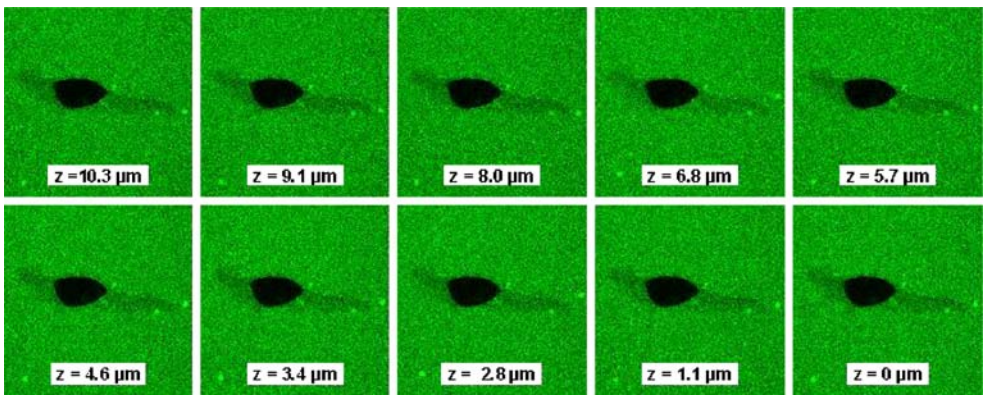

Fig. 6 Confocal microscopy fluorescent images of A2780 human ovarian cancer cells incubated 1 h with FITC-labeled chitosan/γ-PGA nanoparticles with folate (*z*-series, from the *top* of the cell to the *bottom*)

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