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Original article

Interaction of folate – Linked silica nanoparticles with HeLa cells: Analysis and investigation the effect of polymer length

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ABSTRACT

This work is a continuance to our previous findings on silica nanoparticles (NPs) modified with diamine polymer, carboxymethyl-β-cyclodextrin (CM-β-CD) and folic acid (FA), respectively. When four different polymer lengths (D230, D400, D2000 and D4000) were analyzed, the release rate of anticancer agents was inversely related to the polymer length while the cell toxicity was directly related to the length. We investigate here the effect of polymer length on the extent of cellular interaction with HeLa cells. The mean particle size, the polydispersity (PD) and the zeta potential of the NPs were measured using dynamic light scattering (DLS), the quantitative analysis of the extent of NPs' interaction was studied using fluorescence microscopy and transmission electron microscopy (TEM) was used to qualitatively visualize them. The particle size increased by increasing the polymer length, the PD values were within the acceptable ranges (0.3–0.5) and the zeta potential was in the range of (−16 to −20 mV). A direct relation was observed between the fluorescence intensity and the length. All modified NPs were capable of entering the cells, however a greater number of NPs with long polymers was observed compared to short polymers. Thus, the direct relation of polymer length to the cell toxicity is due to the release rate behavior and the enhanced interaction of NPs which possess long polymers.

1. Introduction

Preparing drug delivery systems (DDSs) based on coating inorganic NPs, such as silica, with different polymers has gained a great interest. Such coating can markedly influence and alter different properties of NPs including their solubility, stability, biocompatibility, and dispersibility (Gann and Yan, 2008). In addition, coating NPs with different materials could enhance or retard their interaction with cells or tissues (Behzadi et al., 2017). This will, indeed, make them good potential candidates not only for drug delivery but also for sensors, biomedical devices, electronics, catalysis and optics (Gann and Yan, 2008). Coating nanoparticles with polymers has been performed either chemically or physically (Chithrani, 2015; Glogowski et al., 2006). The chemical attachment, which is more frequent, is achieved through providing strong covalent linkages and often produces materials with enhanced properties, particularly, the stability issues (Mahmoud et al., 2016; Gann and Yan, 2008).

We have previously prepared a DDS based on silica NPs, loaded with a mixture of anticancer agents made of thymoquinone - melatonin (TQ-MLT) and modified, respectively, with diamine polymer, CM-β-CD and FA (Khattabi et al., 2017). Later on, we have evaluated the effect of polymer length on the in vitro properties of this system using four different lengths of the polymer (D230, D400, D2000 and D4000) (Khattabi et al., 2018). In particular, we have examined the effect of polymer length, of this FA-linked NPs, on the drug release rate and the cell toxicity toward human cervical cancer cell line (HeLa cells) which tend to overexpress folate receptors (FR) on their surface (Mansoori et al., 2010). Our findings have demonstrated that the drug release rate decreases with increasing the polymer length. More importantly, a massive burst release was observed with short polymers (D230 and D400) which released most of their drugs within the first hour while a sustained release pattern was exhibited with long polymers (D2000 and D4000)

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(Khattabi et al., 2018). On the other hand, the cell toxicity toward HeLa cells increases by increasing the polymer length (Khattabi et al., 2018). As have been speculated by us, the slow release rate, achieved by long polymers, would most likely provide enough time for the NPs to release most of their drugs once they enter the cells resulting in a greater cell toxicity effect (Khattabi et al., 2018).

However, the goal of the present study is to evaluate if the cell toxicity effect is related not only to the release rate behavior but also to the extent of cellular interaction which involves either cellular attachment and / or internalization. Even though different studies have investigated the effect of other factors on the cellular uptake, such as nanoparticle size, shape and charge (Yu and Zheng, 2016), however and to the best of our knowledge, only few researchers have studied the effect of polymer length on the cellular interaction of folate-linked nanomaterials. The surface modification of inorganic NPs with polyethylene glycol (PEG) and its derivatives has taken the priority in different applications including cancer therapy (Chithrani, 2015). For instance, the effect of PEG length on the targeting ability of folate-linked microemulsions as well as liposomes has been studied and found that this effect is improved by increasing PEG length (Shiokawa et al., 2005; Ohguchi et al., 2008; Gabizon et al., 1999). In other words, long polymers are capable of improving the targeting effect of FA-linked NPs much better than short polymers. As different types of polymers can be conjugated to different types of NPs to prepare different DDSs and each system has its own characteristics, we intend here to particularly study this relation for our system. Moreover, a clear correlation is intended to be established here between drug release rate, cell toxicity, polymer length and cellular interaction.

In this study, fluorescence microscopy was used to quantitatively measure the extent of cellular interaction with HeLa cells while transmission electron microscopy (TEM) was used to qualitatively visualize the NPs. Fluorescence microscopy can be used as a quantitative tool by measuring the fluorescence intensity of different labeled materials (Waters, 2009). This is feasible for our study since the NPs we used here are commercially available; fluorescein isothiocyanate (FITC) - labeled propylcarboxylic acid functionalized silica NPs. TEM is also one of the most reliable methods used for the characterization and tracking of NPs, particularly for metallic NPs due to their high electron density (Bernsen et al., 2015; Costanzo et al., 2017). The tiny structural details of NPs can be visualized in a high resolution manner, after their dehydration (Mourdikoudis and Pallares, 2018). Silica NPs, specifically, have moderate electron density and this allows their appearance as distinguishable spherical particles (Costanzo et al., 2016). Also, through TEM images, a differentiation between the NPs associated to the cell surface and internalized into the cells can be achieved. Unlike TEM, this differentiation will not be easy probed using fluorescence microscopy. However, the main objective here is to study the extent of either cellular attachment or internalization into HeLa cells as a function of polymer length. In addition, the cellular association of the NPs usually precedes their internalizations.

2. Materials and methods

2.1. Preparation of modified silica nanoparticles

The materials, the procedures for (TQ-MLT) loading and the surface modification of the commercially available propylcarboxylic acid functionalized mesoporous silica NPs (particle size 200 nm, pore diameter 4 nm) with diamine polymers (D230, D400, D2000 and D4000), carboxymethyl-β-cyclodextrin (CM-β-CD) and folic acid (FA), were described previously by us, in details (Khattabi et al., 2018; 2017).

2.2. DLS analysis of the NPs

The mean particle size (Z-average), the polydispersity (PD) and the surface charge (zeta potential) of the modified NPs were measured by DLS technique using (Zeta sizernano series, Malvern U.K). Diluted suspensions of the NPs in PBS (pH = 7.4) with a concentration of $4.5 * 10^{-3}$ mg/ml were analyzed. The suspensions were first sonicated for few minutes and the measurements were performed in triplicates with 10 runs and the average was then calculated. The settings of the instrument were adjusted to have a refractive index (RI) = 1.332 for PBS and 1.48 for silica NPs, viscosity = 1.20 cp and temperature = 25 °C.

2.3. Cell preparation for fluorescence microscopy

Hela cells, which were obtained from Sigma Aldrich, were dispensed into 96 well black polystyrene microplate until confluence. The media was then removed and the attached cells were treated, in triplicate, with 0.05 mg/ml of four different samples of modified silica NPs. A sample of HeLa cells alone without any NPs was used as a control. After 6 h of incubation, the media from all samples was removed and the cells washed with phosphate buffered saline (PBS), then fresh media was added. The plate was taken immediately to fluorescence microscope (FLX800TBI, 100 WATTS, biorad) for analysis in the range of (400–600 nm) using the excitation and emission spectra peak wavelengths very close to FITC wavelengths (485 and 538, respectively). For fluorescence microscopy measurements, the intensities of the cells incubated with the NPs as well as the control cells were presented. The intensities were also presented after subtracting the effect of HeLa cells (control).

2.4. Cell preparation for transmission electron microscopy (TEM experiment)

HeLa cells (passage no. 4) were cultured in complete DMEM Medium (10% fetal bovine serum, 1% L- glutamine, 1% penicillin streptomycin and 0.1% gentamycin solution), and incubated at 37 °C for 48 h. The cells were then dispensed into 6 well cell culture plate for 24 h. The media was then completely removed and the attached cells were treated with (0.05 mg/ml) of four different samples of modified silica NPs as well as a sample of unmodified NPs. The samples were first sonicated in HeLa media for few minutes then incubated with the cells for 6 h. A sample of cells was kept untreated and used as a control. After 6 h, the media was removed and the cells were washed to remove the unattached NPs. The cells were then dissociated using Trypsin/EDTA and PBS for 2–3 min. The collected cells were transferred into sterile tubes and centrifuged at 1500 RPM for 10 min. The supernatant was then removed and the cells in each tube were fixed using 200 μl of glutaraldehyde (3%). The samples were then dehydrated through ethanol series, passed through a "transition solvent" such as propylene oxide and then infiltrated and embedded in epoxy resin. After embedding, the resin block was sectioned by ultramicrotomy. Sections of 70-nm thickness were then collected on metal mesh 'grids' and stained with electron dense stains before observation in the TEM (Morgagni FEI 268 with megaview 3 digital cameras, Holand).

3. Results and discussions

3.1. Characterization of the modified NPs using DLS

The successful attachment of diamine polymers, CM-ß-CD and FA was previously confirmed by us, including their mean particle size (Khattabi et al., 2018). However, we have repeated here our

measurements for the size and further examined their polydispersity (PD) and surface charge (zeta potential). As shown in Table 1, the mean particle size of the NPs increased by increasing the polymer length, confirming their successful attachment and it was in the range of (200–392) nm.

Polydispersity is a measurement that provides an indication of the heterogeneity of the particle size in a mixture and samples with PD index values more than 0.7 exhibit usually a very broad size distribution (Nidhin et al., 2008). Our results have shown that the values were within the acceptable range and they were higher for NPs with long polymers (D4000 and D2000) compared to short polymers, as shown in Table 1. This is most probably due to the higher susceptibility of the NPs with long polymers for intermolecular interaction and thus more tendency to form agglomerates.

The zeta potential, which shows the surface charge of the NPs and their potential attraction to the cells, was also measured and the values were in the range of (−16 to −20 mV). This negatively charged range is due to the fact that all samples have FA on their surface and FA has negative groups at pH = 7.4. Generally, the internalization of NPs by the cells depends on their surface charges and positively charged ones are taken up more efficiently compared to neutral and negatively charged NPs (Xiao et al., 2011). This is due to the fact that cellular surfaces have negatively charged sulphated proteoglycans molecules that repel negatively charged nanoparticles (Mislick and Baldeschwieler, 1996). However, certain researchers have concluded that there are few cationic sites on the cell surfaces that are capable of adsorbing and binding to negatively charged particles (Patila et al., 2007). They have speculated that the negatively charged NPs form clusters (agglomerates) when binding at the cationic sites due to the repulsion exhibited by the large negatively charge domains of cell surface (Patila et al., 2007). Furthermore, the bounded NPs on the cell surface cause a reduction in the charge density that may enhance the adsorption of other free NPs.

However, the effect here is not due solely to the charge but more importantly to the targeting effect of FA as well as the polymer length as will be discussed below.

3.2. Quantitative analysis of NPs' interaction with HeLa cells using fluorescence microscopy

In the present study, the interaction of silica NPs modified with four different polymer lengths has been quantitatively investigated in an in vitro cell system using fluorescence microscopy. The NPs used here are the commercially available FITC-labeled propylcarboxylic acid functionalized silica NPs. FITC has excitation and emission spectra peak wavelengths of approximately 495 nm and 519 nm, respectively (Liu et al., 2016). Thus, for fluorescence microscopy, we have used the wavelengths very close to those specific for FITC (485 and 538, respectively).

The goal here was to thoroughly study the effect of polymer length of FA- linked silica NPs on the extent of cellular interaction with FR overexpressing HeLa cells. As has been previously demonstrated by us, there was a direct relation between the polymer length and the percentage of cell toxicity toward HeLa cells, for this particular system, when loaded with a combination of anticancer agents (Khattabi et al., 2018). This relation was due mainly to the inverse relation between the polymer length and release rate behavior which was also observed in the same study. Long polymers (D2000 and D4000) showed slower release rate but greater cell toxicity compared to short polymers (D230, D400). More importantly, for short polymers, most of the drug was released within the first hour with a massive burst release behavior while long polymers showed a sustained release behavior (Khattabi et al., 2018). Thus, long polymers would provide the NPs with enough time to release most of their drugs inside cells resulting in greater cell toxicity. However, the effect of polymer length on the cell toxicity may also due to the fact that when FA linked to long polymers it will associate with and capture its folate receptors much better than short polymers. A similar study, for instance, demonstrated that the association and the cytotoxicity of the folate-PEG_{5000}-linked microemulsion to KB cells were higher than folate-PEG_{2000}-linked microemulsion (Shiokawa et al., 2005). Thus, we intend here to further investigate this fact for our particular system using the fluorescence microscopy to measure the difference in fluorescence intensity of HeLa cells when incubated with these NPs, under the same conditions.

As shown in Fig. 1A, the samples of HeLa cells alone without any NPs (control) have shown a relatively high fluorescence intensity. Even though HeLa cells incubated with the four samples of the NPs have shown differences in their intensities and higher values compared to the control sample, the differences appear insignificant due to the background effect (control). In order to better evaluate the differences, the control sample was then subtracted from the samples incubated with the NPs, as shown in Fig. 1B.

It is obvious now that the fluorescence intensity of HeLa cells increases with increasing the polymer length (D4000 > D2000 > D400 > D230) with clear differences between them (Fig. 1B). The intensities caused by D4000 and D2000 are 8- and 5- fold higher than D400 and D230, respectively. This generally confirms that long polymers interact with the cells much better than short polymers. As this relation is in agreement with our previous result of the cell toxicity effect (Khattabi et al., 2018), we can thus conclude that the greater cell toxicity observed with long polymers for our system is due not only to their release rate behavior but also to the fact that they are capable of interacting with the cells much better than short polymers. These findings demonstrate that linking FA with long polymers will increase its exposure and thus enhance the ability of NPs to interact better with FR compared with short polymers. This is also in consistent with a study which showed that increasing the molecular mass of the PEG from 2000 to 3350 Da enhanced the binding of FA-PEG Grafted Liposomes to FR (Gabizon et al., 1999).

The fluorescence observed here is caused by the NPs either associated with or internalized into the cells. Even though it is not easy to recognize the location of the NPs using this method, we intend here to investigate both the extent of cellular uptake and the attachment of the NPs which usually precedes their internalization. However, to qualitatively visualize the position of the NPs with respect to the cells and as a function of polymer length, several TEM images were collected for this purpose.

3.3. Qualitative analysis of NPs' interaction with HeLa cells using TEM

TEM has been used to qualitatively monitor and visualize the location of the NPs with respect to HeLa cells as a function of polymer length. Several TEM images of HeLa cells, incubated with four different modified silica NPs under the same conditions, were collected to get a general idea about the position of the NPs. As shown in Fig. 2A, untreated cells have their vesicles empty while cells treated with four different modified silica NPs have many of their vesicles occupied by NPs, mainly as small aggregates. This observa-

Table 1
DLS results of the modified NPs with diamine polymers.

Diamine polymer	Mean particle size (nm)	PD, average	zeta potential (mV) ± SD
D230	200	0.34	−20 ± 2.14
D400	253	0.39	−16 ± 2.6
D2000	339	0.59	−18 ± 1.33
D4000	392	0.51	−19 ± 1.06

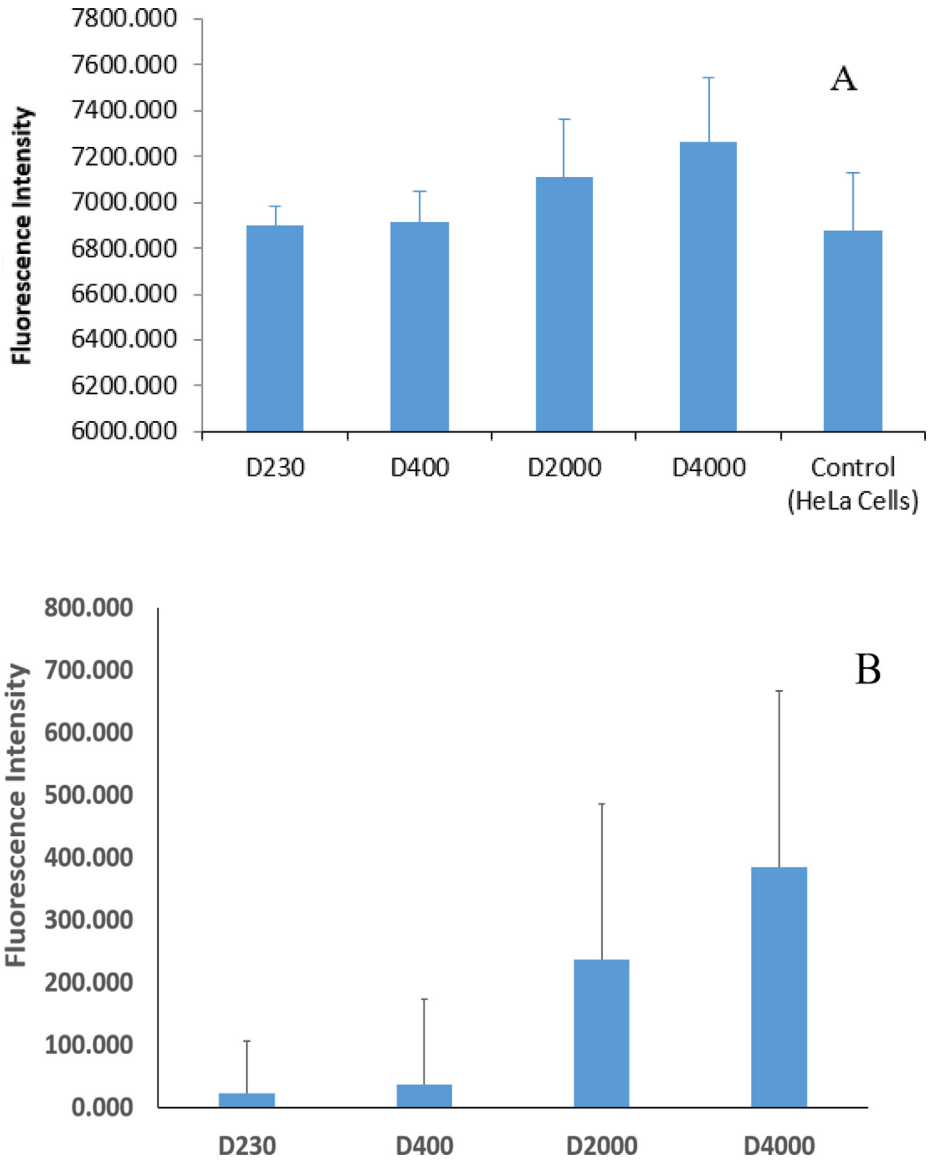

Fig. 1. **(A)** The fluorescence intensity of HeLa cells when incubated for 6 h with four different samples of FITC- labeled silica NPs modified with diamine polymer, CM-β-CD and FA, respectively. The samples differ only in the polymer length where (D230, D400, D2000 and D4000) were analyzed. (B) The fluorescence intensity of HeLa cells when incubated for 6 h with the same samples. The values of the samples shown here are obtained after subtracting the effect (intensity) exhibited by HeLa cells alone (control). Data were shown as ± SD (n = 3).

tion is obvious with all different samples (D4000, D2000, D400 and D230) as shown in Fig. 2 (B, C, D and E, respectively). As this is a qualitative method, it is hard to quantify the amount of internalized NPs for all samples. However, it is interesting to find that NPs modified with both long and short polymers are capable of entering the cells efficiently.

Even though all modified silica NPs, with long and short polymers, have an ability to enter the cells and their quantification was not straightforward here, the extent of their cellular attachment was different and distinguishable. Different regions from each sample of treated cells were scanned and images with a group of cells were collected to visualize the NPs and to get a general estimation about the quantity of the attached NPs. Fig. 3 represents images of HeLa cells treated with unmodified silica NPs (A) and NPs modified with D4000 (B), D2000 (C and D), D400 (E) and D230 (F). Generally, it is obvious here that the amount of attached NPs modified with long polymers (D4000 and D2000), where they attached as small and large aggregates, is much greater than short polymers (D400 and D230). More importantly, cells treated with unmodified NPs has almost none or very few NPs around them. As has been previously mentioned in the experimental part, cells were incubated simultaneously and under the same conditions with these NPs for 6 h then washed to remove the unattached ones and prepared for TEM analysis. Since only loosely attached NPs would be removed after washing, it is clear that most of the attached NPs modified with short polymers were removed after washing and only the internalized part persists inside the cells.

It is worthy to emphasize that the sizes of the NPs were very close as shown from DLS results (Table 1). This narrow range of size distribution would indeed minimize / exclude the effect of the particle size itself on the extent of the cellular uptake. Actually the contribution of the long polymers to the overall size was not great even though at the end they have enhanced the properties of the NPs. Regarding the effect of folic acid density on the cellular uptake, it was also insignificant here as the amount used was the same for all NPs. Based on the experimental part, all NPs were pre-

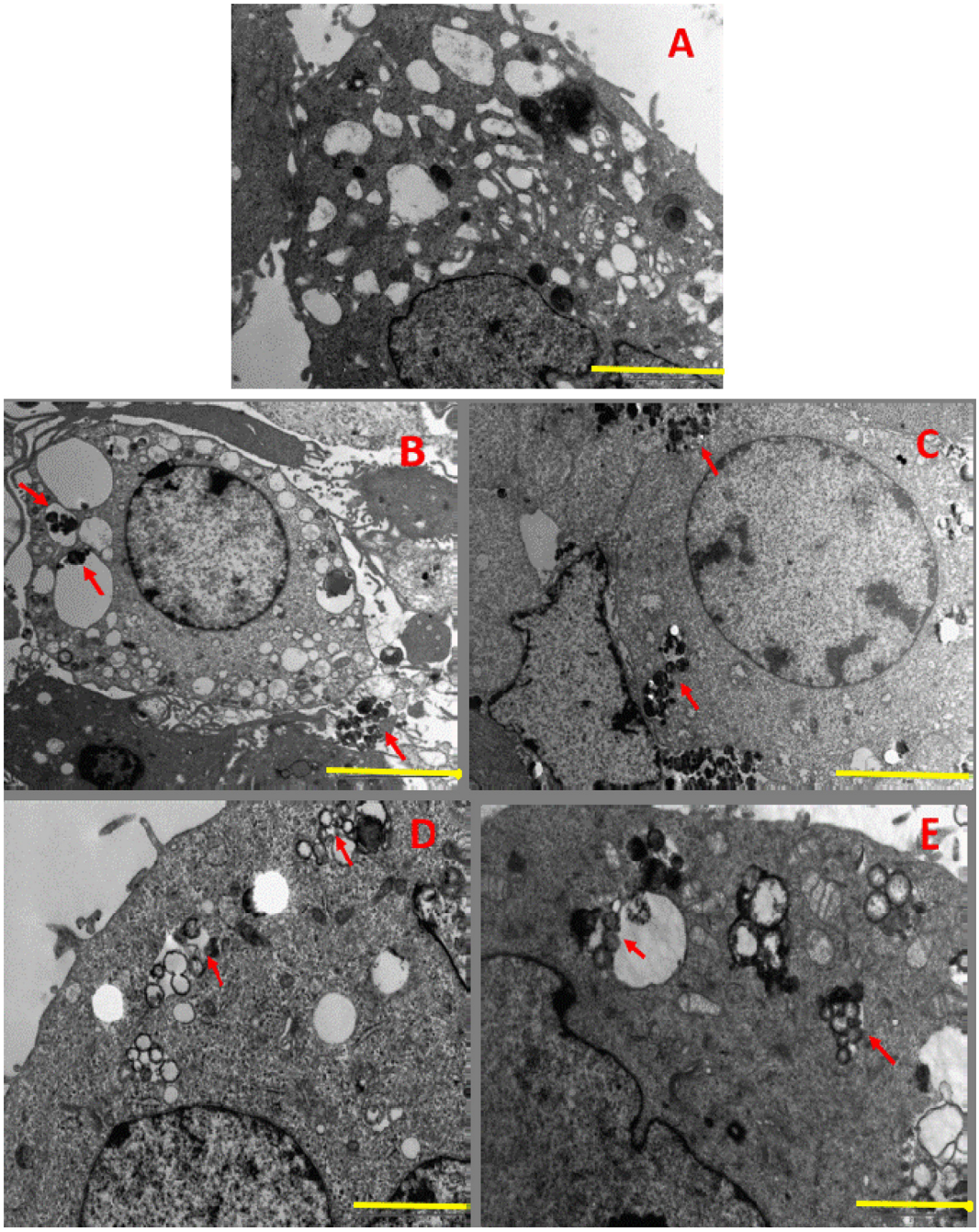

Fig. 2. TEM images of untreated HeLa cells (A) and cells incubated for 6 h with four different samples of silica NPs modified with diamine polymer, CM-β-CD and FA, respectively. The samples differ only in the polymer length where D4000 (B), D2000 (C), D400 (D) and D230 (E) were investigated. All modified samples are capable of entering the cells and persist inside vesicles in the cytoplasm as small aggregates. Scale bar: A, B and C (5 μm), D and E (2 μm).

pared simulatously, under the same conditions and using the same amounts of all reagents. The only difference was the polymer length.

Based on these observations, we can conclude that this specific surface modification (diamine polymer, CM-β-CD and FA) is very efficient in enhancing the cell interaction, both internalization and surface attachment, and long polymers are more efficient than short polymers. Despite the fact that distinguishing the amount of internalized NPs here was not clear, results obtained from the difference in the amount of attached NPs is adequate to prove that when FA is linked to long polymers, the capability of NPs to capture FR and enter the cells will be improved. As the attachment of NPs to the cell surface precedes their internalization and the number of attached NPs with long polymers is greater than short polymers, this will eventually end with more NPs, as well, inside the cells. This conclusion is in agreement with the results obtained from fluorescence microscopy where cells treated with long polymers showed higher intensities than short polymers. Thus, based on results obtained from both fluorescence microscopy and TEM images, one can conclude that the slightly fluorescence intensity caused by short polymers is attributed mainly to the internalized NPs while the relatively high intensity caused by long polymers is due to both the internalized and the attached NPs. More importantly, these findings will further confirm the fact that linking FA to long polymers will enhance the NPs' cell toxicity toward cancerous cells because of the resulted slower release rate caused by long

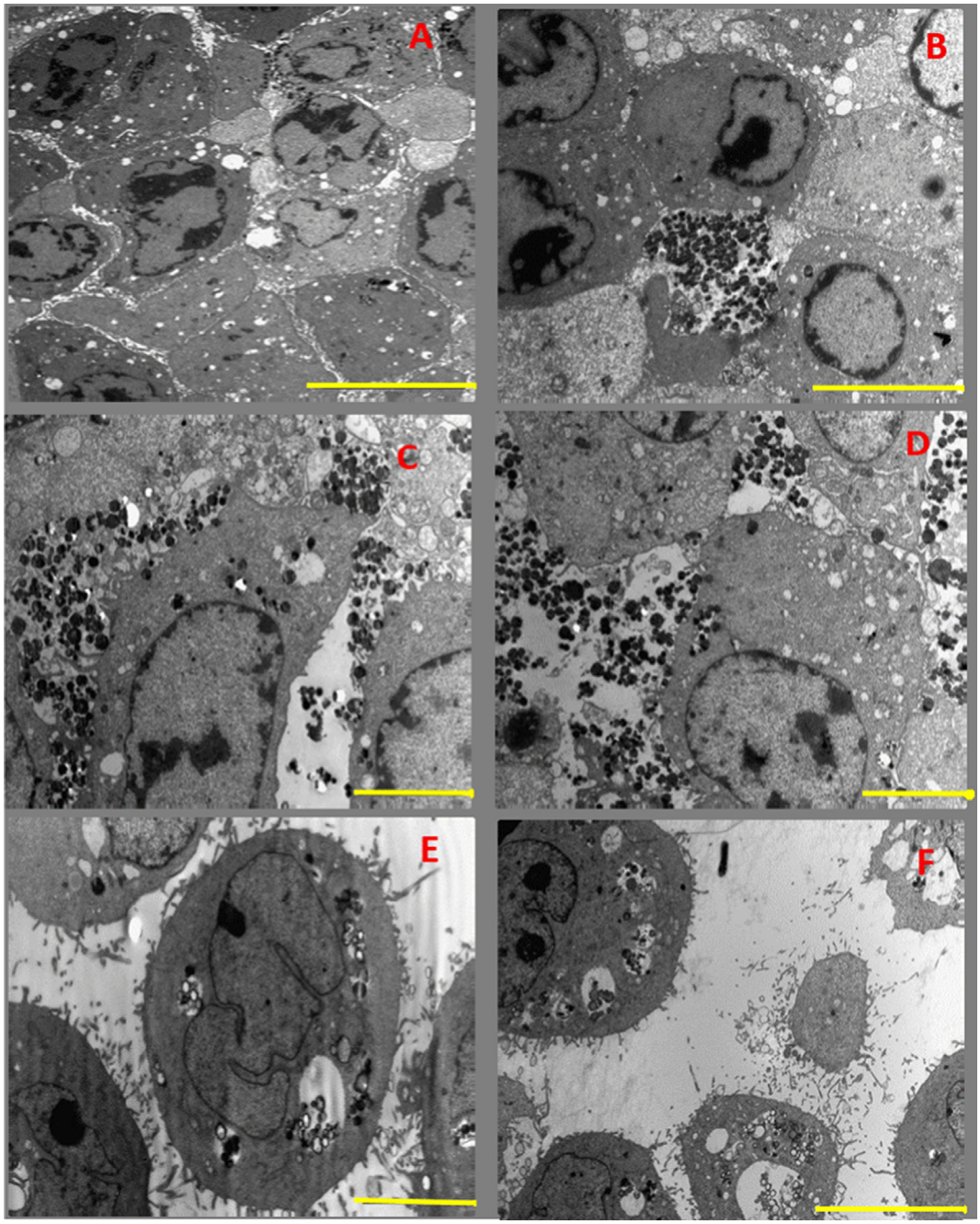

Fig. 3. TEM images of HeLa cells incubated for 6 h with unmodified NPs (A) and with four different samples of silica NPs modified with diamine polymer, CM-β-CD and FA, respectively. The samples differ only in the polymer length where D4000 (B), D2000 (C and D), D400 (E) and D230 (F) were investigated. The number of attached NPs modified with long polymers, as small and large aggregates, is greater than short polymers. Scale bar: A, B and F (10 μm), C, D and E: (5 μm).

polymers compared to short polymers (Khattabi et al., 2018) as well as their better ability to interact with the cells.

Since all modified NPs were capable of entering the cells here, it is important to mention that this process occurred mainly by endocytosis. This is in an agreement with studies which have explored that positively charged NPs were internalized by non-endocytosis pathways while negatively charged NPs were internalized via endocytosis pathways (Cho et al., 2009). Also, it has been suggested that the high cellular uptake of negatively charged NPs in certain studies is attributed to the formation of nanoparticle clusters (agglomerates) (Patila et al., 2007). As shown in Fig. 4 (A and B), once the agglomerates of NPs attached to the cell membrane, they were enclosed by vesicles which then persist in the cytoplasm (Hsiao et al., 2014; Lammel et al., 2019).

4. Conclusions

This work is a continuance to a previous work performed by us on a DDS consisting of silica NPs and modified with diamine polymer, CM-β-CD and FA, respectively. That study investigated the effect of polymer length on the in vitro characteristics of this system where four different polymer lengths (D4000, D2000, D400 and D230) were analyzed. The study confirmed that the cell toxicity of anticancer agents toward HeLa cells increased by increasing the polymer length. This was mainly a result of the slower release rate obtained with long polymers compared to short polymers. In our present work, we investigated the effect of the same polymer lengths of this system on the extent of cellular interaction with HeLa cells. The results obtained here from fluorescence microscopy

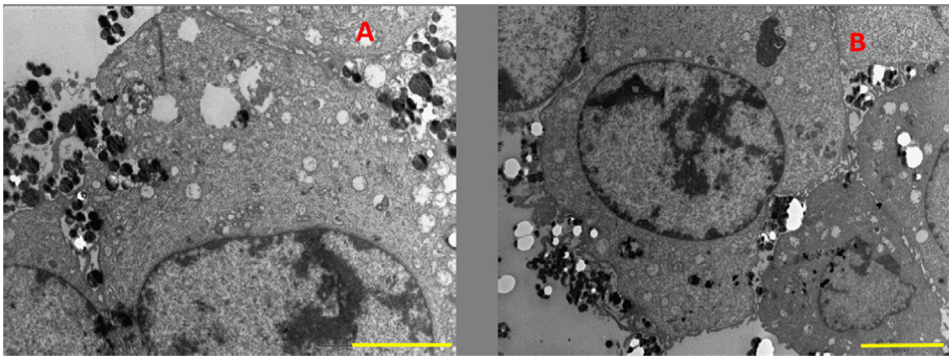

Fig. 4. Enlarged TEM images of HeLa cells incubated with modified silica NPs showing their internalization by endocytosis process. The attached NPs were enclosed by vesicles which then persist in the cytoplasm. Scale bar: A and B (5 μm).

showed that the cells incubated with NPs modified with long polymers had much higher intensities compared to short polymers. TEM images showed that all modified NPs were capable of entering the cells, however, a greater estimated number of NPs modified with long polymers were capable of attaching to the cell surface compared to short polymers. Based on the previous and current results, we concluded that the direct relation of polymer length to the cell toxicity is due not only to the release rate behavior, but also to the fact that long polymers are more capable of interacting with the cells than short polymers for this FA- linked DDS system.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

Areen Khattabi devised the project and the main ideas and wrote the manuscript, Nouf Mahmoud helped in completion the experimental part and revised the manuscript.

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