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Talanta 67 (2005) 486–491
Talanta
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Cytotoxicity of nanoparticle-loaded polymer capsules

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Available online 1 August 2005

Abstract

Cytotoxic effects of micrometer-sized polymer capsules composed out of alternating layers of polystyrenesulfonate (PSS) and polyallylamine hydrochloride (PAH) on a fibroblast cell line have been investigated with an adhesion assay. For the purpose of visualization with fluorescence nanometer-sized CdTe nanoparticles have been embedded in the walls of the capsules. Similar to free CdTe nanoparticles, toxic Cd-ions are also released from CdTe nanoparticles that have been embedded in capsules. At high capsule concentrations, the capsules start to sediment on top of the cells and thus impair cell viability.

Keywords: Cytotoxicity; Nanoparticle; Polymer capsule

1. Introduction

The aim of targeted drug delivery is to selectively deliver drugs to desired parts of a tissue and to release them locally at the target in a way that only the target tissue but not the surrounding tissue is affected by the effect of the drugs [1–3]. Many different drug carrier systems have already been developed and investigated. One concept is to load drugs into the interior of hollow carriers, such as lipid vesicles [4] or polymer capsules [3,5]. In this way, the drug is protected inside the carrier against degradation. Furthermore, the encapsulation of the drug inside a carrier prevents any effect of the drug outside the target tissue. Once the carrier system has reached the target tissue, the carrier has to open in order to release the drug.

Recently, we have suggested a concept for the practical realization of such a carrier system based on polyelectrolyte multilayer capsules in whose shells fluorescent, magnetic and metallic nanoparticles are embedded, on whose surface proteins are adsorbed and whose interior is loaded with drugs [6]. In our concept, such loaded polymer capsules are administered by injecting them close to the target tissue. A magnetic field gradient is then focused on the target tissue. Due to the magnetic particles incorporated in the walls of the capsules, the capsules should be accumulated in the target tissue by trapping them in the magnetic field. Focusing of magnetic particles in magnetic field gradients has already been successfully demonstrated in animal experiments for so-called ferrofluids [7–12]. The proteins adsorbed to the capsule surface should be chosen in a way that they specifically recognize receptors on the cell membranes of the target tissue [1,2,13,14], resulting in specific receptor–ligand-mediated uptake of the capsules by the cells. The whole process of the capsule uptake by the cells of the target tissue could be followed by the fluorescence of the fluorescent nanoparticles that are incorporated in the capsule walls. Once inside the cells, the capsules are irradiated in the infrared at a wavelength that is barely absorbed by the tissue, but which is absorbed by the metallic particles embedded in the capsule shell. By absorption of the infrared light, the metallic particles are heated. We now can envisage two different strategies. By heating the metallic particles to a high degree, the temperature increase will result in a burst of the capsules [15,16] and should finally lead to a destruction of the cell by heat [17,18]. This strategy is know as hyperthermia and has already been demonstrated by several groups [19–24]. On the other hand,

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doi:10.1016/j.talanta.2005.06.042

by moderately heating of the capsules, their shells could be ruptured in a controlled way, resulting in holes through which the drug loaded inside the capsules could be released.

We have to point out that the individual ideas are not new, but the novelty of our concept is the combination of several mechanisms in one single carrier system. So far, one important aspect has been neglected. If capsules are to be introduced as drug carrier systems into animals or human beings, first their biocompatibility has to be proven. Capsules can be assembled out of most polyelectrolytes and therefore it is no problem to compose capsules out of biocompatible materials. Living cells can, for example, be embedded in shells of such polyelectrolytes [25,26]. However, although capsules can be composed out of biocompatible and biodegradable materials, cells still might be affected by their uptake. In addition, the biocompatibility of nanoparticles incorporated in the capsule walls in order to render them functional can be problematic. Iron oxide-based magnetic particles can be highly biocompatible [27] because iron oxide particles that have been incorporated by cells are eventually degraded to iron and oxygen, which are both natural compounds. However, cytotoxic effects of other magnetic particles have been observed [28]. Commonly used fluorescent nanoparticles are based on cadmium-containing materials, such as CdTe or CdSe. It has been reported that upon corrosion, highly toxic cadmium ions are released from these particles, although embedding the particles in appropriate shells can hinder corrosion [29]. Another group has pointed out the influence of the surface chemistry of such particles on the cytotoxicity [30,31]. Even inert gold particles can be cytotoxic under certain conditions [32].

Recently, we have developed a standard assay to investigate cytotoxic effects of nanoparticles on cell cultures [33]. In this report, we applied this assay to investigate the cytotoxicity of polymer capsules and of polymer capsules loaded with fluorescent CdTe particles. As reference, the cytotoxicity of plain CdTe nanoparticles was investigated.

2. Materials and methods

Mercaptoacetic acid stabilized fluorescent CdTe nanoparticles have been synthesized in aqueous solution following standard protocols [34]. In this work, we used particles with an absorption wavelength of the first electronic transition at 530 nm. From this wavelength, the mean diameter of the CdTe particles can be deduced to be 3.0 nm [35] and each CdTe particle exposes around 80 Cd atoms on its surface. Polymer capsules of 5 μm diameter have been composed out of 8–10 alternating layers of polystyrenesulfonate (PSS) and polyallylamine hydrochloride (PAH) [36]. In part of the capsules, fluorescent CdTe nanoparticles were incorporated [6,36]. CdTe nanoparticles, plain capsules and CdTe-labeled capsules were added to NRK normal rat kidney cells [6,33] and cytotoxic effect on the cells were detected using an adhesion assay [33]. For this assay, cells were seeded on a glass substrate, imaged with an automated optical microscope and the number of adherent cells was counted using an image processing software. After the counting – procedure cells were kept in serum-free SATO medium and CdTe nanoparticles, plain capsules or CdTe-labeled capsules were added at different concentrations. After 48 h of incubation, nonadherent cells were removed by rinsing and the adherent cells were again imaged and counted by optical microscopy. An automated *x*–*y*-stage of the microscope allowed for finding the same positions on the cell culture substrate, so that the number of adherent cells before and after incubation with particles could be counted at exactly the same region [33]. As a result, the ratio $R(c)$ of adherent cells after and before incubation with particles with concentration c was obtained. In addition, the viability of adherent cells before and after incubation with particles was measured with a commercial kit (L3224, Molecular Probes) [33].

3. Results and discussion

In this work, we could demonstrate that CdTe nanoparticles, as well as plain and CdTe-labeled polymer capsules, are ingested by NRK cells. Ingestion of nanometer-sized colloidal semiconductor nanoparticles by living cells has first been observed almost 10 years ago [37] and for a general overview, we refer to recent reviews and the references cited therein [38,39]. The uptake of mercaptoacetic acid-coated CdTe nanoparticles investigated in this study was found to be identical to the uptake of mercaptopropionic acid-coated CdSe nanoparticles that has been described in detail elsewhere [33]. Moreover, it has been demonstrated that also micrometer-sized particles can be incorporated by living cells [40]. The cellular uptake of the polymer capsules used in this study (see Fig. 1) has already been reported previously, although by another cell line [6]. The incorporation of particles is nonspecific, i.e. there are no specific ligands present on the surface of the particles.

One potential point of criticism is that fluorescence microscopy images as shown in Fig. 1 are no definite proof that the particles actually have been ingested by the cells. For nanometer-sized semiconductor nanoparticles, it has been shown by confocal microscopy that particles are incorporated by cells [41]. More details can be found in a recent review [39]. For micrometer-sized particles, such as the polymer capsules used in this study, one might argue that they are too big to be ingested by cells, since the height of adherent cells typically is only in the order of a few micrometer, in the case of NRK fibroblasts around 4 μm [42]. However, as can be seen in Fig. 1, cells can adjust their shape and bulge around ingested capsules. Confocal microscopy images show that polymer capsules of 5 μm diameter can indeed be ingested by NRK fibroblasts (data not shown). With conventional microscopy (such as the image shown in Fig. 1), an indication whether capsules are inside the cells or just adsorbed to their outside can be obtained by changing the

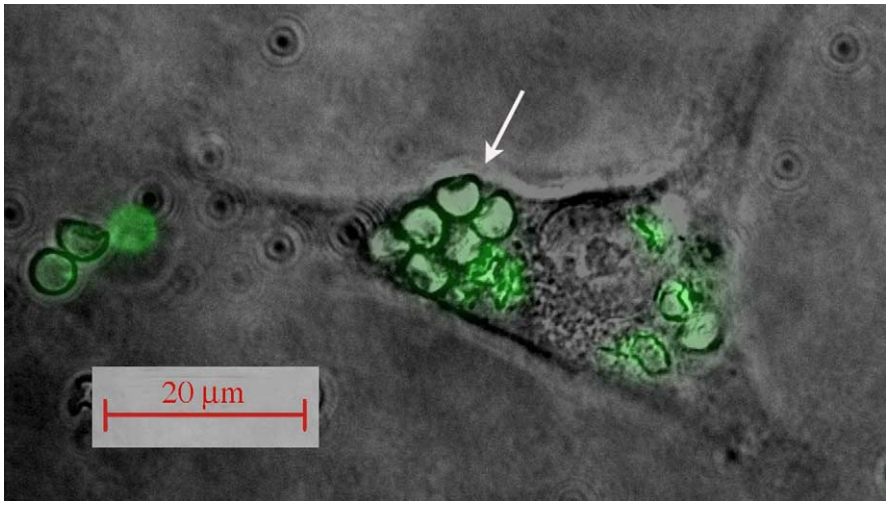

Fig. 1. Overlay of phases contrast and fluorescence image of a NRK normal rat kidney fibroblast cell with internalized capsules. Ingested capsules (labeled with green fluorescent colloidal CdTe nanoparticles) are stored around the nuclei inside the cells. The used capsules of about 5 μm diameter have a polyelectrolyte polymer shell that is terminated with a positively charged polymer. Experimental details about the uptake of capsules by cells can be found in a previous report [6]. The shape of the cell has been deformed by some of the ingested capsules (see white arrow).

focus [6]. Naturally, capsules will be adsorbed to the outside of the cell membrane before they can be ingested. Therefore, upon incubation of cells with capsules, parts of the capsules located at a cell will be only adsorbed to the outer cell membrane, while others already will have been incorporated. It has to be pointed out that in the present study the degree of capsule ingestion has not been analyzed in detail. In general, three species will exist: capsules floating in the medium, capsules adsorbed to the cell membrane and capsules ingested by the cells. Dependent on the incubation time, the equilibrium will shift from free floating to ingested capsules.

In Fig. 2, the results for the adherence assay of CdTe particles are shown. The curves show the same behavior as has been previously reported for CdSe particles [33]. For low particle concentrations, the $R(c)$ value is constant, decreases and increases again at higher particle concentrations to finally reach a constant value for high concentrations. The commercial viability test showed that all adherent cells at low particle concentrations were alive, whereas all adherent cells at very high particle concentrations were dead. This can be interpreted in the following way [33]. Even without addition of CdTe particles, $R(c = 0)$ is smaller than 1 because culture in serum-free medium impairs the cellular development and after 48 h culture in serum-free medium, less adherent cells are present than before. However, serum-free medium had to be used in order to hinder cell proliferation so that the number of cells after and before incubation with particles could be directly compared. For low CdTe concentrations, no additional effect besides the effect of the serum-free culture medium was observable and $R(c)$ stayed constant. Upon further increasing the CdTe concentration, $R(c)$ decreased, i.e. the number of adherent cells decreased. CdTe particles release toxic Cd-ions [29], which lead to an impairment of parts of the cells within this concentration region. Poisoned cells slowly lost contact to the cell culture substrate, detached and the number of adherent cells decreased. Cell adhesion is an active process and for this reason, slow poisoning of the cells results in detachment from the surface. We have to point out that the commercial viability test showed

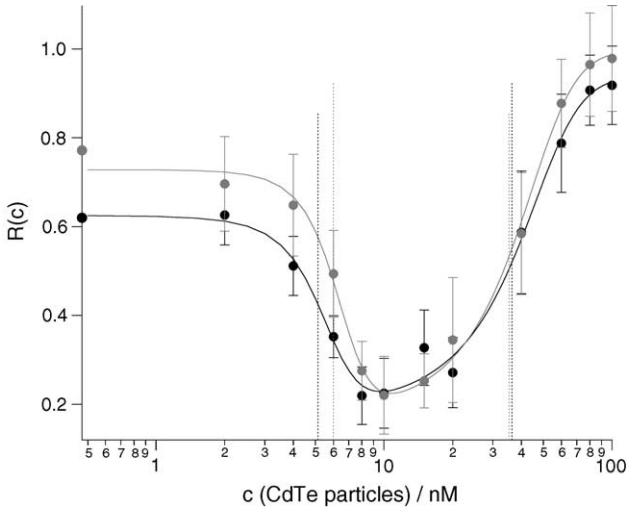

Fig. 2. Ratio $R(c)$ of adherent cells after and before incubation of NRK fibroblasts with mercaptoacetic acid stabilized CdTe particles as a function of the particle concentration c. Values are shown for two independent series of measurements (shown in black and grey). The mean value and standard deviation of each data point has been obtained from 20,000 investigated cells [33]. The data points of each series were fitted with the function $R(c) = a_1 - a_2/(1 + \exp((a_3 - c)/a_4)) + a_5/(1 + \exp((a_6 - c)/a_7))$. The fit results are $a_1 = 0.67$, $a_2 = 0.57$, $a_3 = 6.0$ nM, $a_4 = 1.1$, $a_5 = 0.89$, $a_6 = 36$ nM and $a_7 = 13$ for the series shown in black and $a_1 = 0.57$, $a_2 = 0.45$, $a_3 = 5.1$ nM, $a_4 = 1.1$, $a_5 = 0.82$, $a_6 = 37$ nM and $a_7 = 14$ for the series shown in grey. The a_3 parameter yielded from the fit describes the onset of the decay in the $R(c)$ curve and thus the critical concentration for the beginning of cytotoxic effects. The a_3 parameter of both fits were found to be 6.0 and 5.1 nM, which results in a mean value for the critical concentration of 5.5 ± 0.6 nM.

that all adherent, and thus counted, cells were alive under these conditions. Further increase in the CdTe concentration resulted in an increase of $R(c)$ until a final saturation. The commercial viability test showed that under these conditions, all adherent cells were dead. This suggests that at high CdTe concentrations, the number of released Cd-ions is sufficient enough to kill the cells very fast. Instead of slowly poisoning the cells, which leads to a slow detachment of the cells, at such high Cd concentrations, cells are instantaneously killed and their debris remains on the cell culture substrate surface. These dead cells (=cell debris) are counted as adherent cells, and therefore, $R(c)$ is increasing again. $R(c)$ is even higher for very high CdTe particle concentrations compared to very low particle concentrations. At very high concentrations, cells are instantaneously killed and most of them remain as fragments on the substrate, resulting in a high $R(c)$ value. As reported previously, we have fitted the $R(c)$ curves with a bi-sigmoidal function $R(c) = a_1 - a_2/(1 + \exp((a_3 - c)/a_4)) + a_5/(1 + \exp((a_6 - c)/a_7))$ with seven fit parameters a_1–a_7 [33]. The a_3 parameter of the function is a measure for onset of the decrease in the $R(c)$ function and thus for the beginning of cytotoxic effects of the CdTe particles on the cells. In our study, we found a_3 (CdTe particles) = 5.5 ± 0.6 nM, and thus, this concentration of CdTe particles can be considered as critical concentration for the onset of cytotoxic effects. The use of the fitting procedure thus allows us to extract a quantitative threshold for the onset of cytotoxic effects from our data. Since cytotoxic effects are caused by the release of Cd-ions from the CdTe particle surface for these particles [29], we have converted the concentration of CdTe particles to the concentration of Cd atoms lying on the surface of the CdTe particles [33]. For the CdTe particles of 3 nm core diameter used here, each particle has around 80 Cd atoms on its surface. This results in a critical concentration of a_3 (Cd surface atoms) = 440 ± 50 nM. This is in good agreement with the value a_3 (Cd surface atoms) = 650 ± 120 nM found for CdSe particles in a previous study [33]. We have to point out that the CdTe particles used in this study have been directly synthesized in the presence of mercaptoacetic acid in aqueous solution [34], whereas the CdSe particles used in the previous study [33] have been synthesized in organic solution and then have been transferred to aqueous solution by a ligand exchange procedure in which the original TOPO ligands were partly replaced by mercaptopropionic acid. This independence from the actual synthesis procedure suggests that for these particles, the release of Cd-ions via corrosion of the particles and not an effect connected to the surface chemistry of the particles is primarily relevant for the cytotoxic effects.

For plain polymer capsules, the experimentally obtained $R(c)$ curve has a completely different shape (Fig. 3). $R(c)$ is constant for low capsule concentrations c and starts to slightly decrease at higher concentrations. Viability assays using the commercial kit showed that for all concentrations, all adherent cells were alive. For concentrations of roughly around

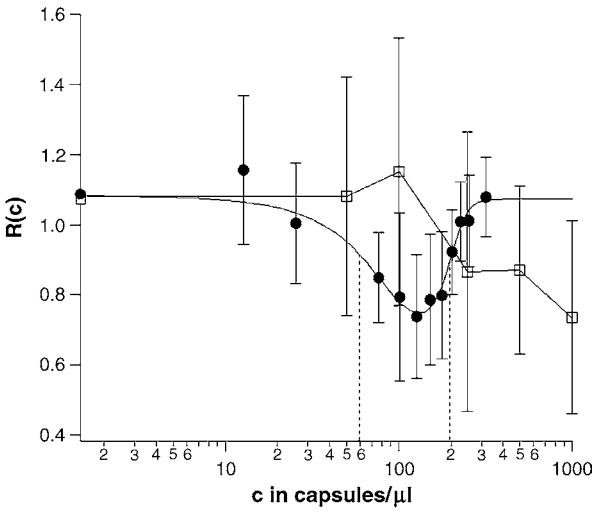

Fig. 3. Ratio $R(c)$ of adherent cells after and before incubation of NRK fibroblasts with plain (shown in open boxes) and CdTe-loaded (shown in black circles) polymer capsules of 5 μm diameter as a function of the capsule concentration c. The mean value and standard deviation of each data point has been obtained from 20,000 investigated cells. The data points of the series using CdTe-loaded capsules were fitted with the function $R(c) = a_1 - a_2/(1 + \exp((a_3 - c)/a_4)) + a_5/(1 + \exp((a_6 - c)/a_7))$. The obtained parameters are: $a_1 = 1.1$, $a_2 = 0.43$, $a_3 = 60$ capsules/μl, $a_4 = 27$, $a_5 = 0.38$, $a_6 = 190$ capsules/μl and $a_7 = 25$. The a_3 parameter of the fit was found to be $a_3 = 60$ capsules/μl. At this capsule concentration, clear cytotoxic effect for the CdTe-labeled capsules can be seen, whereas for plain capsules, no decrease in $R(c)$ can seen at least until concentrations of 100 capsules/μl. The concentration of 100 capsules/μl corresponds to 10 capsules in the incubation medium per cell.

100 capsules/μl, no effects of the capsules on the cells are visible (Fig. 3). Higher capsule concentrations lead to a slow impairment of the cell viability, which eventually results in cell detachment. Microscopy images showed a massive precipitation or sedimentation of capsules on the cell surface and on the cell culture substrate (Fig. 4). We assume that this sedimentation is responsible for the impairment of the cells. A similar effect has been observed for the sedimentation of polymer-coated nanoparticles on cell surfaces in a previous study [33].

The embedding of CdTe particles in the wall of polymer capsules leads to an $R(c)$ curve similar to that of free CdTe particles (Fig. 3). Application of the commercial viability test showed that adherent cells were alive for low capsule concentrations (before the dip in the $R(c)$ curve) and dead for high capsule concentrations (after the dip in the $R(c)$ curve) similar to the case of cells incubated with free CdTe nanoparticles (Fig. 2). These findings suggest that in the case of CdTe-loaded polymer capsules, the release of Cd-ions from the CdTe particles is responsible for cytotoxic effects. The wall of the CdTe nanoparticles-containing capsules is permeable for ions and thus does not offer protection again the release of Cd-ions from the surface of the CdTe nanoparticles. However, the CdTe particles are embedded inside the walls in a way that they cannot be released from there as a whole. By comparing the critical concentrations a_3 for the cases of

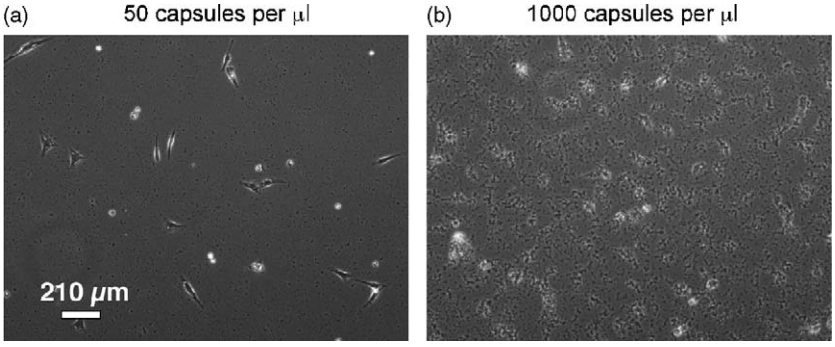

Fig. 4. Phase contrast microscopy image of adherent NRK fibroblast to which plain 5 μm diameter polymer capsules have been added at concentrations of: (a) 50 capsules/μl and (b) 1000 capsules/μl. For high capsule concentrations, massive sedimentation of capsules on the surface of the cells and the cell culture substrate is visible.

free CdTe particles and CdTe-labeled capsules, we can derive an estimate on how many CdTe nanoparticles are embedded in each capsule. Cytotoxic effects for CdTe-labeled capsules started at $a_3 = 60$ capsules/μl (Fig. 3). This corresponds to a critical capsule concentration of $a_3 = 60$ capsules $(\mu l^{-1})/6 \times 10^{23}$ capsules $(mol^{-1}) = 1 \times 10^{-16}$ M. For free CdTe nanoparticles, a critical particle concentration of $a_3 = 5.5 \times 10^{-9}$ M was found (Fig. 2). Thus, we can estimate that roughly 5.5×10^{-9} M/1×10^{-16} M = 5.5×10^{7} CdTe nanoparticles (with a core diameter of 3 nm) are embedded in the shell of each capsule (with a diameter of 5 μm), assuming that the pathway of cytotoxic effects is the same in both cases.

In summary, we have shown that polystyrenesulfonate/polyallylamine hydrochloride multilayer capsules do not show a detectable effect on cell viability in case cells are only incubated with a few capsules per cell. However, at high concentrations, capsules start to sediment on top of the cells and thus impair cell viability. Nanoparticles of different materials can be embedded in the capsule walls to provide functionality. Since capsule walls form a porous network of polyelectrolyte layers, they are highly permeable for small molecules, such as inorganic ions. Therefore, toxic ions released from the nanoparticles embedded in the capsule walls due to corrosion can diffuse into the medium. For this reason, cytotoxicity of these nanoparticles has to be considered. For fluorescent mercaptoacetic acid-coated CdTe nanocrystals, cytotoxic effects are visible above a critical particle concentration of 5.5 nM. Accordingly, capsules comprising CdTe nanoparticles exhibit higher toxic effects than plain capsules. For this reason, the nanoparticle systems, which are to be embedded into the capsules, have to be chosen very carefully. Instead of mercaptoacetic acid-coated CdTe particles, for example, silica-coated CdSe/ZnS nanoparticles could be used, which are far less cytotoxic [33].

Acknowledgements

This project has been founded by the Volkswagen Foundation (I80/051-54, ALR, GBS, WJP), by the Emmy Noether Program of the German Research Foundation DFG (WJP) and by the Fond der Chemischen Industrie (ALR, WJP).

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