Neuron Cells Uptake of Polymeric Microcapsules and Subsequent Intracellular Release

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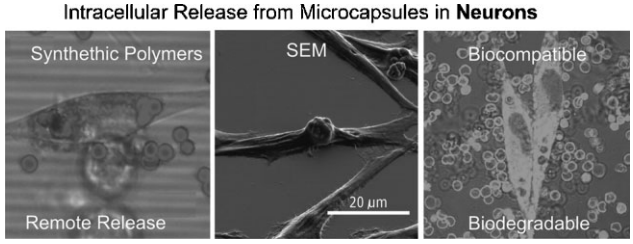
Neuron cells uptake of biodegradable and synthetic polymeric microcapsules functionalized with aggregates of gold nanoparticles incorporated into their shells is demonstrated in situ. In addition to traditionally used optical microscopy, electron microscopy is used both for higher-resolution imaging and for confirming the uptake by focused ion beam cross-sectioning of specific cells in situ. Subsequently, physical methods of release are compared to chemical methods wherein laser-induced intracellular release of dextran molecules into the cytosol of hippocampal neuron cells is studied in comparison to biodegradation. Implications of this work for neuroscience, bio-medicine and single cell studies are discussed.



Introduction

Recent advances in nanotechnology permit the design of drug delivery vehicles with increased level of complexity and functionality. It is difficult to overestimate its role in developing proper delivery containers, through optimization and functionalization of the exterior shell, control loading and release functions. Therefore, in the last decade, advanced drug delivery techniques saw a rapid growth of a broad range of functions ranging from in vitro studies to sophisticated in vitro applications. In vitro studies, which are mostly performed on cellular cultures, are considered as the first step before developing in vivo applications. In this area intracellular trafficking of proteins, peptides and biomolecules, in general, is one of the most important direction in the field.

Intracellular trafficking of proteins and peptides is an important functionality of living cells and living organisms. For example, they govern protein folding, kinetics of protein and peptide interaction as well as transport of peptides to the cell surface. As such, it is of high interest to develop systems, tools and methods for following intracellular transport of proteins and small peptides. Although extensive statistical analysis is typically applied to a large number of cells, the area of single cell studies is also gathering popularity. That is because researchers can monitor cells, albeit a smaller number, with much better quality. Indeed, such an approach provides a principally different type of information. Instead of monitoring thousands of cells, single cell studies permit to analyze fewer cells but incur complete information about their state at any point in time.

Recently, we have demonstrated[1] intracellular delivery and controlled release of small peptides (SIINFEKL), which bind to the MHC Class I proteins, presumably in the endoplasmic reticulum (ER), forming the protein–peptide

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complex. The complex was then observed to present at the cells surface – an important condition in immunology for T-cells activation and attack of infected cells. This knowledge is of fundamental importance to immunology. In regard to neuroscience, intracellular delivery and transport can reveal important information on neuron activity. Inducing neuron[2] activity either triggered or spontaneous is an intense field of research. Currently, neuron internal activity is generally triggered using perfusion micropipette systems which can be rather elaborate, may damage cell membrane and can also lead to leakage of solution into extracellular environment. Therefore, it is desirable to load bio-molecules of interest with minimal interference to cell activity and to release[3] these bio-molecules with high precision in terms of quantity, temporal and spatial location.[4] Doing that non-destructively, i.e. without affecting metabolism of living cells, and without damage to the cell membrane would open extensive opportunities in terms of studying important intracellular processes and neuron network signaling. On the other hand, such approaches and methods can be further applied for in vivo. Polyelectrolyte multilayer capsules[5–7] can withstand intracellular pressures[8] and delivery bio-molecules intracellularly. A number of different cell lines were used in previous studies: MDF-MB-435S cancer cells,[9] stem cells,[10] monkey kidney (VERO-1),[11] Chinese hamster ovary (CHO) and VERO[1] cells, human colon cancer,[12] and HeLa cells.[13] For VERO cells different protocols were used for internalization: prolonged incubation (over 15 h)[11,12] or a specific electroporation procedure.[1]

In this work we demonstrate methods based on polyelectrolyte multilayer capsules as delivery carriers applicable for intracellular delivery of molecules into hippocampal neuron cells. A study of uptake of microcapsules by neurons of polyelectrolyte microcapsules is carried out using conventional confocal laser scanning microscopy (CLSM) and, in addition, advanced electron microscopy: scanning electron microscopy (SEM) technique. We further carry out intracellular release using two principally different methods: an infrared laser radiation and biodegradable self-induced release and discuss potential implications of these methods for delivery and neuron cell functions. These methods are analyzed in regard to their application to neurons as well as from the point of view of application of different but somewhat complementary approaches. Applications of our technique to single cell studies are also discussed.

Experimental Section

Materials

SiO_2 colloidal particles with the mean diameter 4.99 μm were obtained from Microparticles GmbH, Germany. Poly(diallyldimethylammonium chloride) (PDADMAC, molecular weight 200–350 kDa), poly(sodium 4-styrenesulfonate) sodium salt (PSS, molecular weight ≈70 kDa), Poly-L-Arginine hydrochloride (PLA molecular weight 15–70 kDa), Poly-L-Lysine hydrobromide (PLL, molecular weight 30–70 kDa), glutaraldehyde, dextran sulfate sodium salt (DS, molecular weight ≈100 kDa), Rhodamine-B Isothiocyanate (TRITC)-labeled dextran (molecular weight ≈4.4 kDa), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), 20 nm colloidal gold nanoparticles, sodium hydroxide (NaOH), hydrofluoric acid (HF) and salts were obtained from Sigma–Aldrich, Germany. Fluorescein isothiocyanate–dextran (FITC-dextran, molecular weight 4 kDa) was purchased from Fluka. Green dye DiOC18(3) was obtained from Invitrogen Ltd.

Microcapsules Preparation

Two types of capsules were assembled: synthetic for controllable remote release and those formed with bio-degradable polymers; in both cases the Layer-by-Layer (LbL) technique was used for assembly on sacrificial templates.

Non-biodegradable capsules

SiO_2 particles with mean diameter of 4.99 um were used as templates. 2 mg · mL^{-1} solutions of PDADMAC and PSS in 0.5 M NaCl were used as positively and negatively charged polyelectrolytes, respectively, for LbL assembly. Pre-aggregated 20 nm gold nanoparticles were embedded into the shell at the 6th layer.[14a] Pre-aggregation was done by mixing stock solution of gold colloid with 1 M NaCl, as described in previously reported work. After depositing eight polyelectrolyte layers, SiO_2 templates were dissolved with 0.3 M HF (hydrofluoric acid). Dissolution was followed by several washing/centrifugation steps until pH of the supernatant reached values higher than 6. After constructing the capsules, TRITC-labeled dextran was encapsulated by heat-shrinking method according to the previously reported procedure.[1] Briefly, the microcapsule dispersion and 1 mg · ml^{-1} dextran solution were mixed for 10 min and then incubated at 65 °C for 10 min. After cooling at room temperature, excess dextran was washed away by centrifugation/re-dispersion. The distribution of gold nanoparticles or the surface filling factor of microcapsules with gold nanoparticles was chosen to allow for non-destructive intracellular release,[1,14] and it was checked by means of transmission electron microscopy (TEM) using a JEOL-JEM 2010 transmission electron microscope.

Biodegradable capsules

For experiments with biocompatible microcapsules the shells were assembled around $CaCO_3$ particles (approximately 3–5 μm in diameter), which were prepared by mixing solutions of 0.33 M of $CaCl_2$, 0.33 M of Na_2CO_3, together with 1 mg · mL^{-1} solution of FITC-dextran at 1:1:1 proportion (for co-precipitation) upon vigorous stirring. PLA and DS (2 mg · ml^{-1}) solutions in 0.15 M NaCl were used as polyelectrolytes for building 4-bilayer polyelectrolyte shells. After depositing the layers the templates were dissolved with 0.2 M EDTA.

Cells and Cellular Uptake

B50 rat neuronal cells were obtained from European Collection of Animal Cell Cultures (ECACC, Porton Down, UK). These cells[15] are

an ethylnitros urea induced tumor cell line with neuronal morphology. The cells were cultured in the Dulbecco's minimum essential media (DMEM, Sigma 51435C) supplemented with 10% fetal bovine serum (10% FBS, Sigma F2442) and penicillin–streptomyosin (1%), at 37 °C and 5% CO_2/95% air.[16] More specifically, cells were cultured in a 35 mm μ-Dish (Ibidi GmbH) which permits simultaneous observation and irradiation by laser. Once the desired cell density was achieved, the culture medium was changed to the 1% FBS solution to stop cell division.

For incorporation inside cells microcapsules were first transferred into a cell buffer (1% FBS) by re-dispersing them in the buffer solution and then introduced into the cell medium. In case of biocompatible capsules the initial concentration is typically quite high. Therefore, thorough washing and subsequent dilution was used to adjust desired concentration. Washing also allows for removing excess of capsules. For experiments with laser-induced release the capsules were introduced in the evening and left overnight for uptake. Uptake was confirmed by CLSM and SEM with focused ion beam etching at FEI Quanta 3D FEG scanning electron microscope. Ion beam etching was used in situ during the SEM investigations to expose the specific region of specimens and provide clear evidence of uptake.

To visualize microcapsules inside cells, the latter were fixed and stained, and confocal images of microcapsules inside the stained cytoplasm were taken. For SEM investigations, the cells were loaded with $CaCO_3$ particles coated by polyelectrolyte shells. After overnight incubation, cells were fixed with glutaraldehyde, sputtered with carbon and investigated with SEM microscope at acceleration voltage of 5 kV (measurements were done using Everhart–Thornley detector). In control experiments performed by CLSM and conducted together with SEM imaging, microcapsules with encapsulated TRITC-dextran were used, while cells were stained with the green dye DiOC18(3).

Irradiation and Release

The setup and optical scheme used for controlled remote release experiments were similar to those described in previous work.[9,14a,17] A laser diode (830 nm) with incident power of up to 50 mW was used for release experiments; images were acquired by a Leica TS microscope equipped with a 63× (numerical aperture 1.4) oil immersion microscope objective lens. The microscope stage was placed inside the incubation box (37 °C) to expedite time-consuming imaging of living cells.

Results and Discussion

Polyelectrolyte multilayers are extremely versatile due to flexibility in their assembly. This flexibility, as well as nanometer thickness of each layer[9] (and therefore opportunities to nano-engineer desired properties), permit incorporation of various molecules, materials and inorganic particles into the layers. A particularly attractive constituent of microcapsule walls is inorganic nanoparticles. Indeed, they can be used (when assembled in a non-aggregated state[14b]) for enhancing mechanical properties,[18] act as absorption centers for inducing release[19–21] inside cells or can serve as sensors. Some of the most promising applications of polyelectrolyte multilayer capsules involve intracellular delivery and time specific release.

In regard to release, noble metal nanoparticles can be used as active absorption centers controlling the permeability of polyelectrolyte multilayer capsules. In this case the local permeability of microcapsules is affected by (nanometer size area) heating. The best method of visualization of nanoparticles in the walls of polyelectrolyte multilayer capsules is TEM. Figure 1 (a) shows a typical TEM image of a hollow polyelectrolyte multilayer capsule before encapsulation of molecules in its interior.

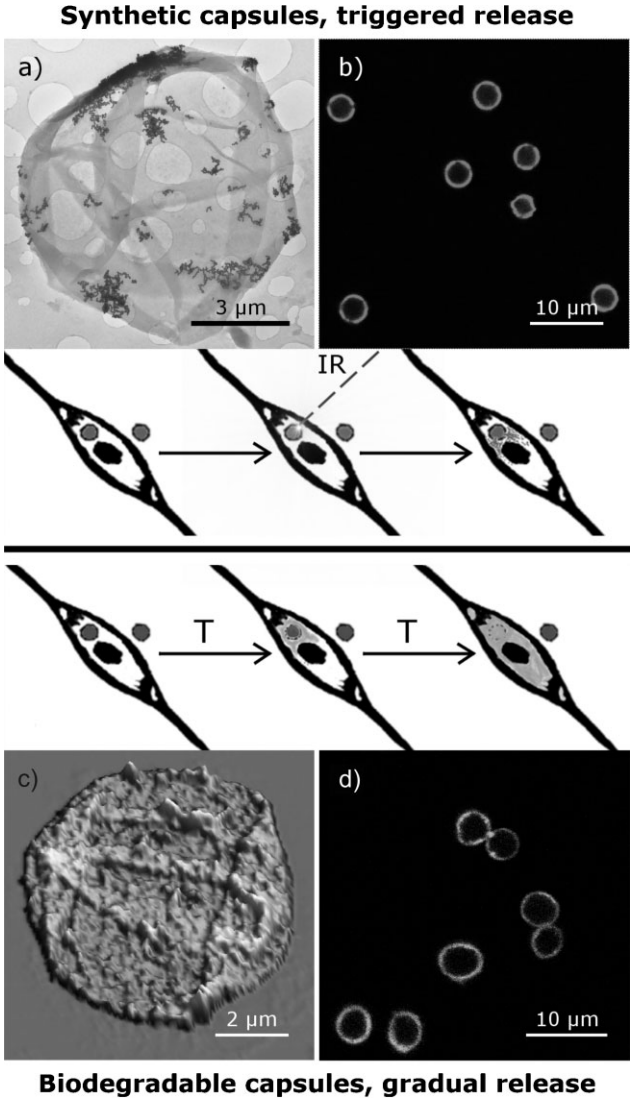

Figure 1. (a)–(b) Microcapsules composed of synthetic polymers: a)TEM, empty capsules, b) CSLM image of microcapsules after TRITC-dextran encapsulation. (c)–(d) Microcapsules composed of biodegradable polymers: c) AFM of a dried microcapsule, d) CSLM image after FITC-dextran encapsulation.

Gold nanoparticles were incorporated in the middle of the eight polyelectrolyte multilayers; they serve as absorption centers for near-infrared laser. Affecting the permeability of the polymeric membrane by nanoparticles-laser interaction is due to localized heating around the nanoparticles.[22] Typically, gold nanoparticles possess a well-pronounced surface plasmon resonance peak around ≈520 nm. Aggregation of nanoparticles provides a red-shift of this surface plasmon absorption due to dipole–dipole interaction on neighboring nanoparticles.[14b] Although anisotropic nanoparticles, for example nanorods,[23] also provide strong near-IR absorption, poor biocompatibility of surfactants can limit their application. Conversely, aggregating nanoparticles (for example, by salt in case of citrate stabilized nanoparticles[14a]) is an effective method in achieving desirable distribution and properties. Figure 1 (b) shows filled microcapsules.

Microcapsules composed of biodegradable polymers such as polypeptides and polysaccharides are optically looking similar to those composed of synthetic ones. An AFM image of a microcapsule is presented in Figure 1 (c), where one can clearly see a pancake-like structure of a dried microcapsule. One of the methods of encapsulation in biodegradable microcapsules is incorporation of materials during the fabrication of the core or post-loading. Figure 1 (d) shows a CLSM image of microcapsules containing FITC-dextran in their interior. It can be noted that these microcapsules exhibit more irregular shapes due to biocompatible templates on which they are constructed. In this work we describe various methods of characterization of microcapsules and compare optical remote release inside neuron cells with that conducted through biodegradable shell of microcapsules. Schematic of experiments is presented in the middle of Figure 1.

Uptake of microcapsules inside cells is one of primary processes for monitoring intracellular cargo trafficking. It can take place, for example, by phagocytosis[24] or it can be induced by electroporation.[1] In the former case, only a limited number of types of cells, i.e. those that exhibit phagocytic activity, can be used. In the latter case, the technique can be applied to many different cell types. Although such a process induces perturbations to the cell membrane, cells recover without affecting their metabolism. We used SEM in order to shed light on microscopic details of this process. In situ focused ion beam milling was used to cross-section cells inside the microscope for studying internalization of the capsules. For this experiment, intact $CaCO_3$ particles possessing polymeric shells were introduced into the cell chamber. These particles are quite rigid, and therefore provide a strong contrast for SEM. Rigid capsules are quite bulky inside the collapsed cell structures. However, it appears that despite of relatively large size (3–5 μm) and rigidity of the core, capsules are internalized by the cells without significant disruption of the cell membrane. Figure 2 (a) shows a SEM image of a cell with a particle inside. This cell was subsequently cross-sectioned by milling away half of the cell and the particle with the focused ion beam. Figure 2 (b) shows the same place after ion beam milling, with a rectangular trench of material removed. The trench extends down into the glass substrate. Figure 2 (c) shows a close-up image of a Figure 2 (b) section depicted by the white dashed rectangle. The cross-section of $CaCO_3$ core can be clearly seen in Figure 2 (c), and it demonstrates the characteristic porous structure[25] of the core. The cell membrane can be seen extending around the capsule, thus confirming that cells can internalize relatively large and rigid objects.[1] It can be also mentioned that fabrication of micrometer capsules is more facile than assembling of nanometer-sized colloidal particles and shells.[26] CLSM images also confirm microcapsules uptake by cells, Figure 2 (d–f). Cells were observed to internalize a large number of capsules; therefore, adjusting the concentration of microcapsules can be used to control a number of uptaken capsules. It was also found in our studies that capsules are confined to the soma of a cell, although in some cases they were found in the axon hillock. The presence of the capsules does not

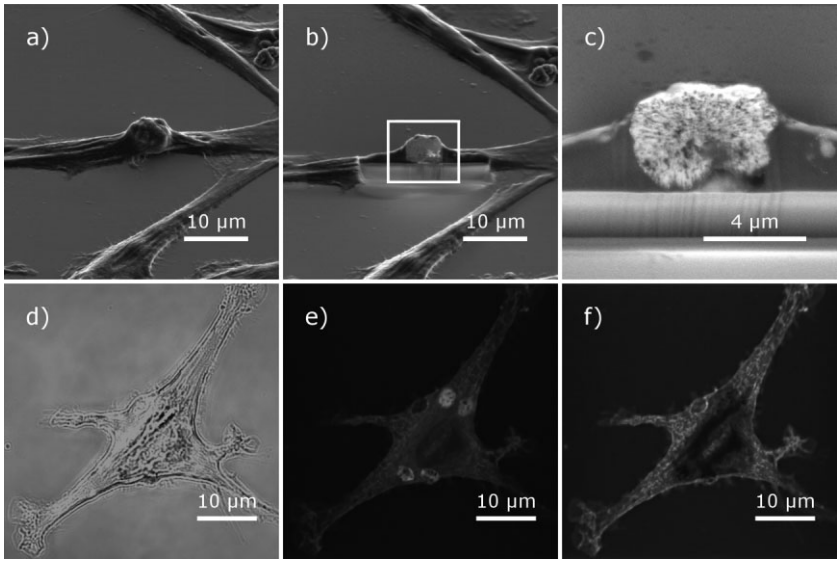

Figure 2. SEM images of a microparticle inside a neural cell: a) a bulge on a cell, b) this bulge lanced by ion beam milling; c) a magnified region of b) showing porous $CaCO_3$ core. Confocal microscope image of an internalized capsule: d) transmission image of a neuron cell; e) the red channel depicting capsules (contour of the cell can be seen through cross-talk from the green channel), and f) the green channel showing contour of the same cell.

seem to affect cell viability on the timescale of our experiments (up to 72 h); this is consistent with previously reported results.

There are a number of methods which can be used to induce release from microcapsules including light,[19–21,17] magnetic particles,[27] ultrasound[28] and biodegradability.[29,30] Comparing biodegradability (chemical and biochemical) with physical methods of release (light, magnetic particles, ultrasound) it can be remarked that latter provide better control of kinetics. Indeed, in the case of biodegradable release by redox-oxidation of disulfide bonds[12] the release may occur even during the uptake. Although this property can be used for in vivo applications, better control is certainly achieved with physical means of release. In the case of enzymatic degradation[11] identifying the zero-point of release is also challenging.

We have chosen light for controlled release from microcapsules due to good control over kinetics of release from microcapsules. Experiments with remote release from microcapsules were performed with TRITC-dextran filled microcapsules with shells functionalized with aggregates of gold nanoparticles. Upon irradiation by a near-IR laser microcapsules release encapsulated materials inside the cells. One of specific details of this approach is that cell membranes are largely unaffected by the laser with wavelength in this spectral range while the absorption of gold nanoparticle aggregates embedded into the shells is tuned to absorb in this spectral region. As a result, we were able to open microcapsules without damage to the cell walls; this is consistent with previously reported results.[1] Images of cells with irradiated capsules are presented in Figure 3. A control experiment without opening microcapsules is demonstrated in Figure 3 (a). The CLSM image of capsules inside a cell immediately after release is shown in Figure 3 (b). It can be observed that dextran spreading out of the capsule rather uniformly. In Figure 3 (c) a thin spring-jet of fluorescing dextran is formed, apparently, along the filament of the cell. Parak and coworkers [31] as well as other groups[1,3,32] investigated cells viability. It was shown that for more explosive-like opening of the capsules possessing high concentration of nanoparticles, the incident laser power on the order of hundred of milliwatt leads to cells damage;[31] that can be applied to photothermal therapy treatment. It can be noted that the main advantage of laser-induced is a well-specified zero-time point of release of encapsulated materials as it allows monitoring release with precision of minutes.[1] Therefore, such experiments are well-suited for tracing kinetics of intracellular release and following intracellular movement of molecules.

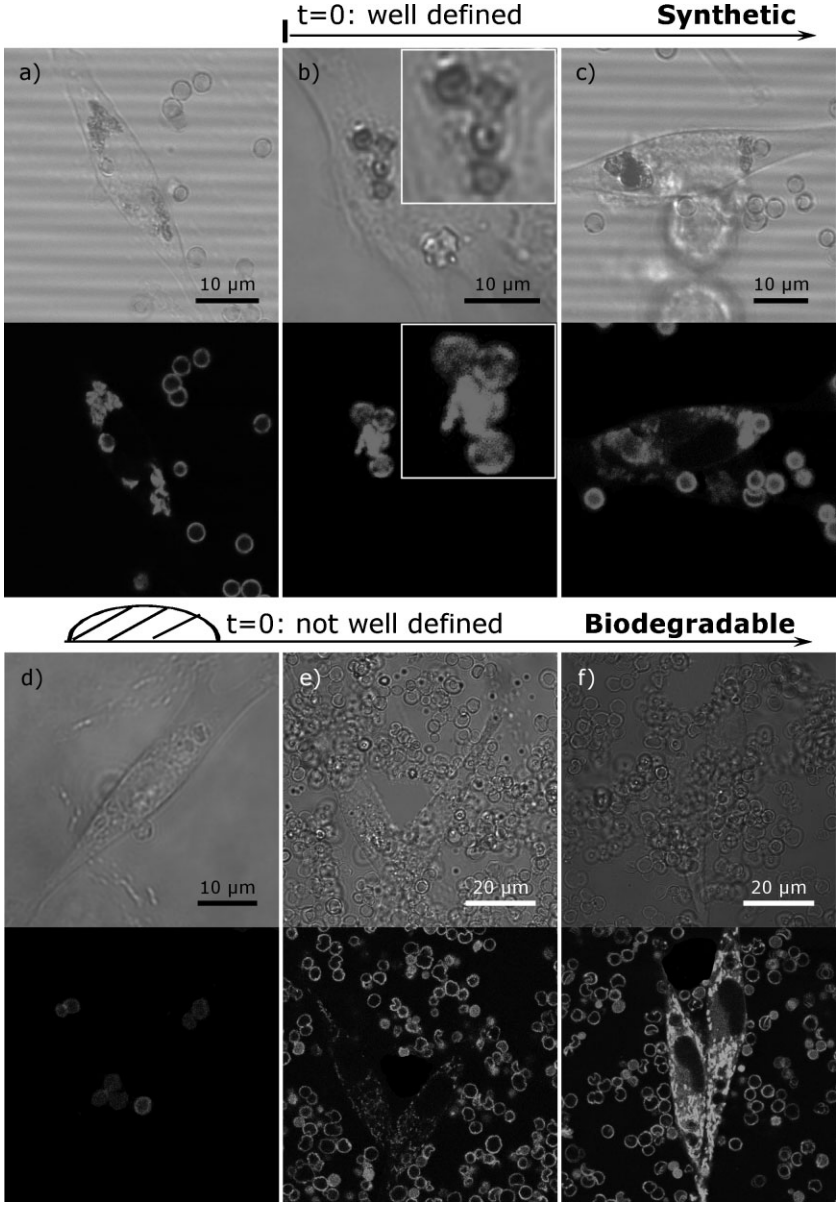

Figure 3. CLSM images of cells with capsules inside after laser treatment: a) microcapsules without exposure to laser light are presented as a control experiment; b) immediately after irradiation, b) 4 h after irradiation. The transmission channel is shown on top and the red fluorescence channel depicting the signal from TRITC-dextran is included right under. CLSM images of biodegradable capsules in cells d) 4 h, e) 8 h, f) 16 h after uptake. The transmission channel is shown on top and the green fluorescence channel depicting the signal from FITC-dextran is included right under.

$CaCO_3$ templates were chosen for constructing microcapsules since they are more biocompatible[33] than those templated on SiO_2 which require HF for dissolution. Encapsulated FITC-labeled dextran was chosen as a model system for these experiments. Figure 3 (d–f) show CLSM images of microcapsules in the cells at various periods of time. First, microcapsules inside cells can be seen after approximately 4 h of incubation, Figure 3 (e). After 16 h the intracellular signal is even more pronounced. It was noticed that the uptake of microcapsules composed of biodegradable polymers is not as active as that in the case of microcapsules composed of synthetic polymers. Since the latter microcapsules are much stiffer[8] it can be interpreted that cells internalize more readily stiffer objects. Time sequence of release from microcapsules can be seen in Figure 3. It can be noted that, unlike the case for remote release performed by laser, experiments with biodegradable microcapsules lack a clear zero-time point of release, thus impeding observation of dynamics of intracellular release. On the other hand, these microcapsules constructed on biocompatible templates using bio-degradable polymers are better suited for in vivo studies.

Further work on microcapsules using neurons can concern neuron delivery,[34] imaging[35] as well as studying cell signaling, injury and recovery,[36] as well as substrates[37,38] for cells[39] including those with nanoparticles.[40,41] Also, using direction-specific release[42] and anisotropic[43] and anisotropic multicompartment[44] capsules it should be possible targeting cellular organelles. We also note that cell culture studies reported in this work will be useful for developing delivery systems for in vivo applications and studying their response.

Conclusion

In this study we reported results of studying intracellular incorporation of microcapsules and microparticles by neurons. It is unequivocally demonstrated that hippocampal neuronal cells, which play an important role in learning, long term memory and spatial orientation, uptake both bio- and non-biodegradable capsules. Thus, our work demonstrates a possibility to use microcapsule technology for payload delivery and release inside neuron cells. Uptake of polymeric microcapsules by neuron cells is demonstrated in situ, and imaged by focused ion beam SEM. Intracellular release is initiated by two principally different methods: time defined remote release by laser light and spontaneous biodegradation of polymeric shells.[45] It is shown in our work that an advantage of the light induced intracellular release is a well-controlled dynamics of the release, whereas advantages of the latter method include potential applications in vivo where external triggering can be problematic. Studies of this neuronal cell line should enable further extension of application of microcapsule technology to cell signalling applications. Another significance of this studies in that anatomically, the hippocampus is an elaboration of the edge of the cerebral cortex and therefore can be potentially relatively easily accessed with the laser radiation in vivo. The results of our work are envisioned to open new opportunities for neuron cell research in the areas which concern intracellular delivery, intracellular dynamics or delivery in vivo. In this emerging area of research multicompartmentalization and multimolecule delivery are expected to be next challenges.[46]

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