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Cell-Specific Integration of Artificial Organelles Based on Functionalized Polymer Vesicles

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ABSTRACT

Cell organelles are subcellular structures characterized by specific functionalities. They often consist of membrane-delineated microcompartments with a unique set of enzymes. Here we report the design of synthetic organelles based on nanometer-sized polymer vesicles, show their introduction into cells in a target-specific fashion, document their intact biochemical functionality in the cellular environment, and study their intracellular trafficking. This novel paradigm of introducing polymer-based artificial organelles to specific target cells for expansion of their biochemical capabilities appears suited for biomedical applications such as enzyme replacement in genetic diseases or, more generically, to add a desired biochemical function to a cell.

Malfunction at the molecular and cellular level is the primary cause of most diseases. This being the case, our current medical instruments are extremely crude. Even the finest scalpel or chemotherapeutic drug is like an elephant in a china store, breaking things right and left. It is only because, unlike china, cells and tissues can heal from injury that modern medicine works at all. To fill the urgent need for much finer therapeutic tools that would enable highly specific medical intervention at the cellular and molecular level, we must develop subcellular scale systems with specialized functionalities that are stable and compatible with living tissues.

Cell organelles are membrane-delineated intracellular components with specialized functions. They have unique biochemical microenvironments, characterized by a specific set of enzymes, and are enclosed by a selective membrane, allowing the subcellular spatial organization of biochemical functionality and decoupling of separate molecular pathways. They are thus responsible for crucial cellular activities, including photosynthesis, biomolecule synthesis, modification and folding of new proteins and lipids, energy production, degradation of macromolecules, and translation of RNA into proteins.[1]

The ability to design artificial cell organelles for specific tasks, to introduce them into cells, preferentially in a cell-specific manner, and to retain their biochemical functionality would add a novel paradigm for medical therapy. The rapid emergence of technologies for the fabrication of structures at the nanometer scale sets the stage for the development of such tools. In particular, nanometer-sized polymer vesicles, self-assembled in water from synthetic biocompatible amphiphilic copolymer building blocks, show promise as platforms for complex subcellular systems as well as tools for the delivery of drugs, enzymes, nucleotides, and diagnostic agents.[2,3]

Promising candidates for in vivo therapeutic use are vesicles composed from the triblock ABA copolymer poly(2-methyloxazoline)-*b*-poly(dimethylsiloxane)-*b*-poly(2-methyloxazoline), PMOXA-PDMS-PMOXA. These copolymers self-assemble in aqueous solution to form highly stable, closed vesicles with a controlled diameter of typically less than 200 nm.[4] Furthermore, they are innocuous in cell culture,[5] show very limited nonspecific plasma protein binding owing to the outer protein-repellent PMOXA layer,[5] and low uptake by macrophages of the reticuloendothelial system in liver and spleen, allowing vesicles to avoid detection by the immune system and consequently prolonging their blood circulation lifetime.[6]

Using this group of amphiphilic triblock copolymers, we and others have shown the feasibility of building polymer-based nanostructures,[4] cell-specific targeting of cells by such objects,[5] their equipment with functional enzymes,[7,8] the transfer of molecules across these membranes using bacterial

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pore proteins,[9] the possibility of switchable biochemical functionality within such objects,[10,11] and their nontoxicity after uptake into cells.[5] Polymer vesicles can stably encapsulate hydrophilic substances, allowing the application of these vesicles as a carrier system for a wide variety of therapeutic agents. It was demonstrated that the vesicle membrane could be modified in different ways. For example, trans-membrane proteins such as bacterial channel proteins can be integrated into the vesicle membranes and can function in the release of therapeutic molecules[8] or the passage of substrates and products into and out of the vesicles.[11] Additionally, by coupling various ligands to the outer vesicular layer, vesicles were shown to specifically interact with target cells expressing the corresponding receptors.[5] A subsequent step involved the formation of functional catalytic assemblies by loading vesicles with multifunctional biological components such as pore proteins and enzymes. Indeed, when loaded with catalytic molecules, functionalized polymer vesicles selectively processed different substrates in vitro.[11]

In continuation, we now present the design of a prototype PMOXA-PDMS-PMOXA artificial organelle based on certain design principles. These include a size suited for cellular integration, a polymer membrane as a diffusion barrier for separation of the microenvironment from the surroundings while allowing substance transfer of the chosen model substrate, an enzyme content allowing a specific biochemical reaction inside the organelle that can be observed from the outside (e.g., by fluorescence microscopy), protection of the content from undesired interference like proteases and inhibitors, and finally, lack of toxicity through choice of biocompatible materials. In the experimental section, we examine the cellular uptake, intracellular distribution, and activity of the polymer vesicles inside target cells, for which we chose macrophages because they are clinically important cells with key functions in both innate and adaptive immunity as well as in diseases such as atherosclerosis,[12] cancer,[13] and autoimmune disorders.[14]

Class A macrophage scavenger receptors are characteristic of activated tissue macrophages and are responsible for cellular uptake of chemically modified low density lipoproteins (LDL) such as acetyl or oxidized LDL.[15] Indeed, surface-functionalizing polymer vesicles with oligonucleotide polyguanylic acid (polyG), recognized by macrophage scavenger receptors, targets them specifically to macrophages.[5] In a first step, the functionalized polymer vesicles (fPVs) were loaded with fluorescence tagged bovine serum albumin (BSA) to generate protein-containing vesicles (BSA-fPVs) either functionalized with polyG or left without ligand.

To further test the cell-targeting properties of the ligand-functionalized protein-containing vesicles and their ability to specifically deliver their content into the target cells, we incubated several cell types, including fibroblasts (Figure 1J), muscle cells (Figure 1K), and hepatic endothelial cells (Figure 1L) with coated BSA-fPVs. After one hour of incubation, we observed no cellular uptake of the BSA-fPVs by any of these cell types. We then incubated THP-1 derived macrophages for one hour in the presence of coated BSA-fPVs (Figure 1D), uncoated BSA-fPVs (Figure 1E), or free fluorescence-labeled BSA (Figure 1F). Only coated BT-fPVs were taken up by approximately 30% of macrophages. Interestingly, coincubation of THP-1 macrophages with coated BSA-fPVs and green-fluorescent acetylated LDL (AcLDL-Alexa Fluor 488) revealed that only cells that internalized acLDL could take up fPVs (Figure 1G−I). These results confirm the target-specific and receptor-dependent nature of fPVs cellular uptake and suggest that coated fPVs target and enter only subpopulations of macrophages, consisting of cells that are actively taking up modified LDL.

Next, we followed the distribution of fPVs inside cells. This was done by coincubating macrophages with green fluorescent organelle selective dyes for the lysosomes, endoplasmic reticulum (ER)/Goi, and the mitochondria, together with fPVs containing red fluorescent dye. Colocalization of fPVs with cytoplasmic organelle-selective dyes would show as yellow and absence of colocalization as green. In addition, a nuclear stain was used to selectively stain the nucleus blue. Using this approach combined with confocal microscopy imaging in live cells revealed that fPVs are internalized by macrophages within five minutes post incubation. FPVS are internalized through the vesicular uptake system because 10 min after incubation they concentrate in lysosomes (Figure 2A,B). One hour later, fPVs reached the ER and Golgi apparatus (Figure 2C−F) but not the mitochondria or nucleus (Figure 2G−J). These results demonstrate that fPVs are taken up rapidly by target cells and localize exclusively to the cytoplasm where they are trafficked to specific sites, such as the ER and Golgi.

To assess the intracellular stability of internalized functionalized polymer vesicles, we performed the calcein release assay. FPVS were loaded with a self-quenching concentration of green fluorescent calcein and incubated for a maximum of 48 h in the presence of macrophages. Calcein would fluoresce only if its concentration drops below the self-quenching threshold due to fPV disintegration or calcein release, i.e., the rising fluorescence slope indicates a minimum lifetime of the vesicles. Limited signal was observed by fluorescent microscopy 12 h after the beginning of incubation (Figure 3A), increasing gradually over the next 36 h (Figure 3B,C), implying that this kind of polymer vesicles are remarkably stable, retaining structural integrity for as long as 48 h inside cells (Figure 3D).

Because specific, compartmentalized enzyme activity ("bioreactor" functionality) is the hallmark of organelles, we loaded polymer vesicles with trypsin, an intestinal serine protease produced in the pancreas. As an exogenous substrate for encapsulated trypsin inside the vesicles, we used the hydrophobic tripeptide serine protease specific substrate BZiPAR (bis-(CBZ-Ile-Pro-Arg)-R110). BZiPAR (Molecular Probes, R6505) is a derivative of rhodamine 110 that comprises a peptide covalently linked to each of R110s amino groups, thereby suppressing both its visible absorption and fluorescence. BZiPAR can cross the membranes of the cell and the polymer vesicles owing to its hydrophobicity, and upon enzymatic processing by trypsin, this nonfluorescent bisamide substrate is converted to green fluorescent rhodamine

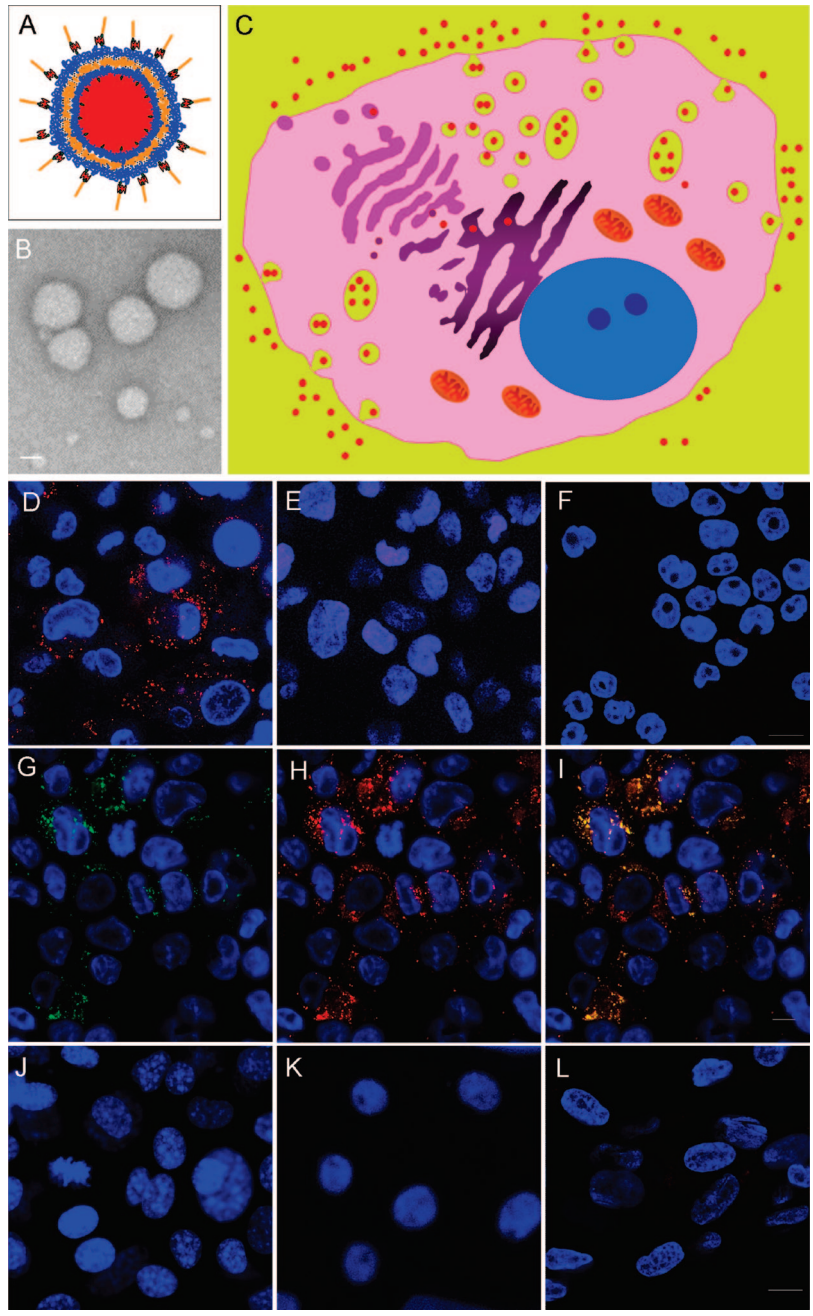

Figure 1. Functionalized polymer vesicles (fPVs; (A) shows a schematic drawing, (B) shows a transmission electron microscopy image of the vesicles, scale bar 50 nm) are specifically internalized by cells. (C) A diagram showing a cross section through a cell incubated in the presence of fPVs loaded with red fluorescent BSA-TRITC (BSA-fPVs). (D) Activated THP-1 macrophages take up BSA-fPVs, but not free BSA-TRITC (E) nor nonfunctionalized BSA-fPVs (F). (G) Green fluorescent AcLDL-Alexa Fluor 488 is taken up by activated THP-1 cells when incubated for 1 h. (H) The same cells also take up BSA-fPVs. (I) Overlay of (G) and (H). Mouse embryonic fibroblasts (J), rat skeletal muscle cells (K), and liver endothelial cells (L) were incubated with BSA-fPVs but did not take them up. Scale bar, 10 μm. The data represent at least three independent experiments. All images were acquired with constant microscope settings (laser power, filters, detector gain, amplification gain, and amplification-offset). Images were acquired with a Zeiss LSM 510 confocal unit.

110. This model allows us to test the principal characteristics of the enzyme-containing polymer vesicles. Namely, that the membranes of the nanoreactors allow the selective diffusion of substrate molecules while retaining the catalytic components, protecting them from degradation and permitting substrate processing to take place inside the nanoreactors. Moreover, members of the serine protease class of peptidases specifically process and activate a wide range of substrates, including enzymes, signaling proteins, and pro-hormones and as such are of special interest to us and could be part of a future therapeutic system based on nanoreactors functioning as artificial cell organelles. THP-1 macrophages were incubated in the presence of polymer vesicles loaded with trypsin (Try-fPVs) for one hour. After washing away noninternalized Try-fPVs, we treated the cells with BZiPAR and hydrophilic protease inhibitors to prevent nonspecific processing of the substrate by lysosomal proteases. Cells containing empty fPVs exhibited only very limited fluores-

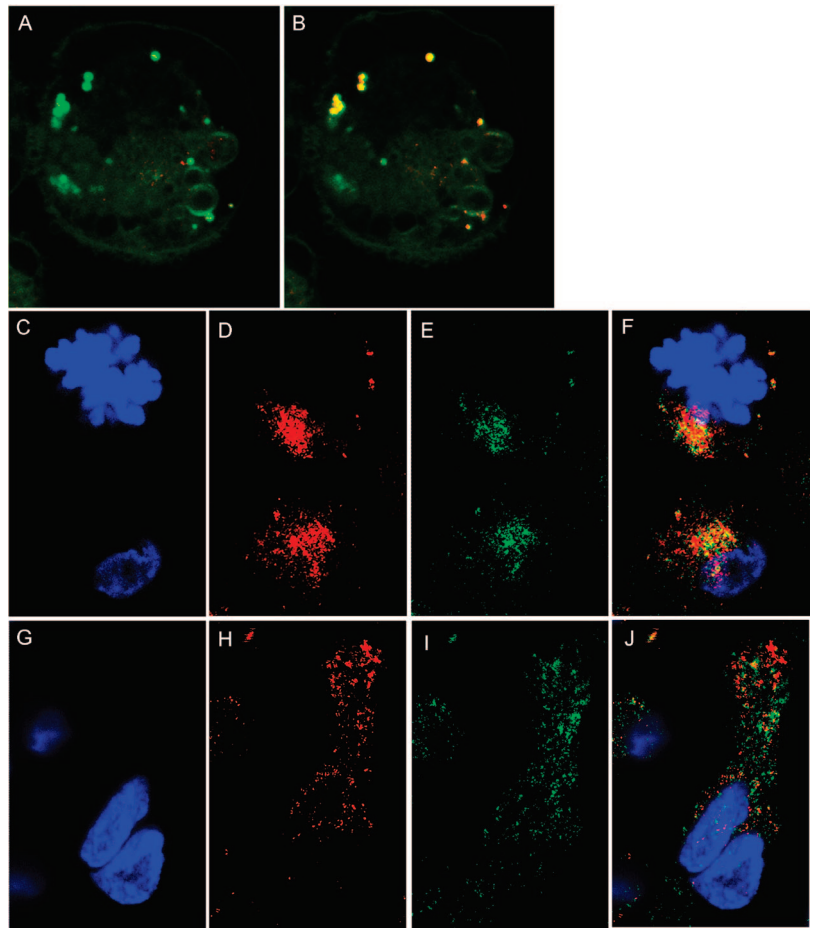

Figure 2. Internalization and intracellular distribution of functionalized polymer vesicles (fPVs) in THP1 cells. Cells were incubated in the presence of fPVs loaded with the red fluorescent dye sulforhodamine B for up to 1 h and stained with one of several organelle selective dyes. (A) A representative cell imaged live and incubated in the presence of fPVs (red) for 5 min and stained with the lysosome specific stain (green), (Lysosensor DND-189, 1 μmol/L). (B) The same cell after additional 5 min of incubation shows fPVs localization in lysosomes (yellow). (C−J) Representative cells incubated in the presence of fPVs (D,H) for 1 h and subsequently stained with nuclear stain (Hoechst 33342, 2 μmol/L) (C,G) and either (E) a Golgi apparatus and endoplasmic reticulum stain (Brefeldin A BODIPY FL conjugate, 36 nmol/L) or (I) a mitochondria specific stain (Mito-Tracker Green FM, 10 nmol/L). (F) An overlay of (C) to (E). Yellow areas indicate colocalization of fPVs (red) with the Golgi apparatus and endoplasmic reticulum (green). (J) An overlay of (G) to (I) shows very limited localization of fPVs to mitochondria. Scale bar, 10 μm. The data represent at least three independent experiments. All images were acquired with constant microscope settings (laser power, filters, detector gain, amplification gain, and amplification-offset). Images were acquired with a Zeiss LSM 510 confocal unit.

cence (Figure 4B,D), whereas cells containing trypsin-loaded fPVs displayed strong green fluorescence in the cytoplasm (Figure 4A,C), suggesting that the substrate entered and was processed inside the fPVs.

A prerequisite for the development of target-specific subcellular therapeutic systems is an understanding of their behavior and function in cells. Here we analyzed essential characteristics such as targeting specificity, intracellular localization, stability, and activity of fPVs. The ability to seek out target cells among a multitude of other cell types would be an essential characteristic of any future target-specific subcellular therapeutic system. In cell culture experiments presented in this work, internalization was confirmed to be specific, allowing fPVs to target specific cell types. Moreover, only macrophages actively taking up acLDL internalized fPVs, suggesting that their uptake can be made specific to cellular subpopulations with certain activity states. Such subpopulation specificity could be highly valuable, for instance, in atherosclerosis, where numerous activated macrophages infiltrate the atherosclerotic plaques. Work is ongoing in our laboratory to test the specificity of fPVs in vivo.

An additional issue of concern in assessing the potential of fPVs to function as subcellular therapeutic systems or artificial cell organelles is their cellular distribution. For instance, evading the destructive environment of the lysosomes would be crucial for processing or synthesizing factors that are susceptible to lysosomal degradation. Here we see for the first time that fPVs can also distribute to the Golgi and ER. Further work is required to elucidate the mechanism by which fPVs reach these organelles. However as fPVs are stable inside cells for many hours, it is possible that a certain proportion may reach the Golgi and ER through transport vesicles that recycle material between the late endosome and the trans-Golgi network. Vesicles composed of triblock copolymers are more stable and less permeable in vitro than liposomes due to the higher length of the hydrophobic block of the polymer, slower dynamics, and intermolecular steric

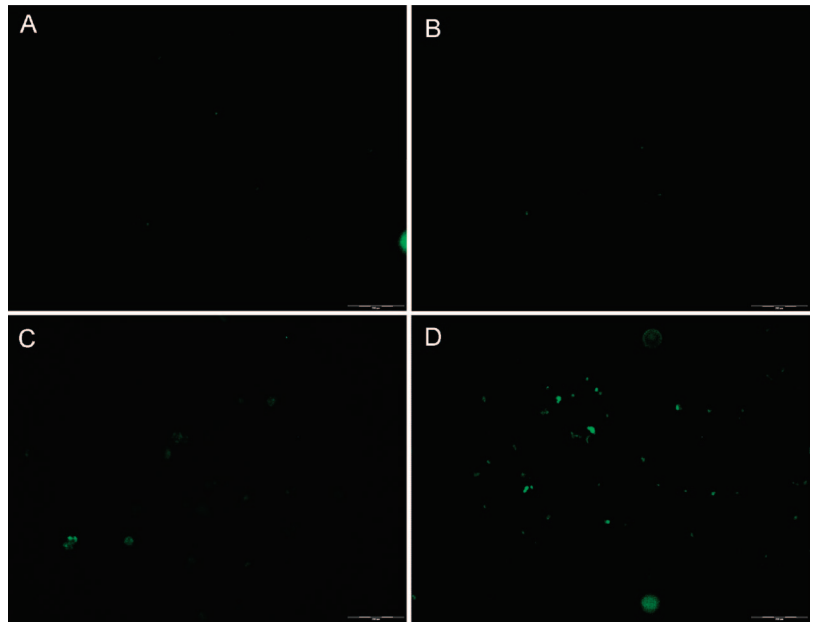

Figure 3. Functionalized polymer vesicles (fPVs) are stable intracellularly for up to 48 h. (A−D) Cells were incubated in the presence of fPVs loaded with a self-quenching concentration of 50 mmol/L green fluorescent calcein. No fluorescence was observed after incubation for 2 h (A) and 24 h (B). (C) Only after 36 h incubation was some fluorescence observed, suggesting that calcein loaded fPVs retain structural integrity for at least this time period. (D) More pronounced fluorescence was observed after 48 h incubation.

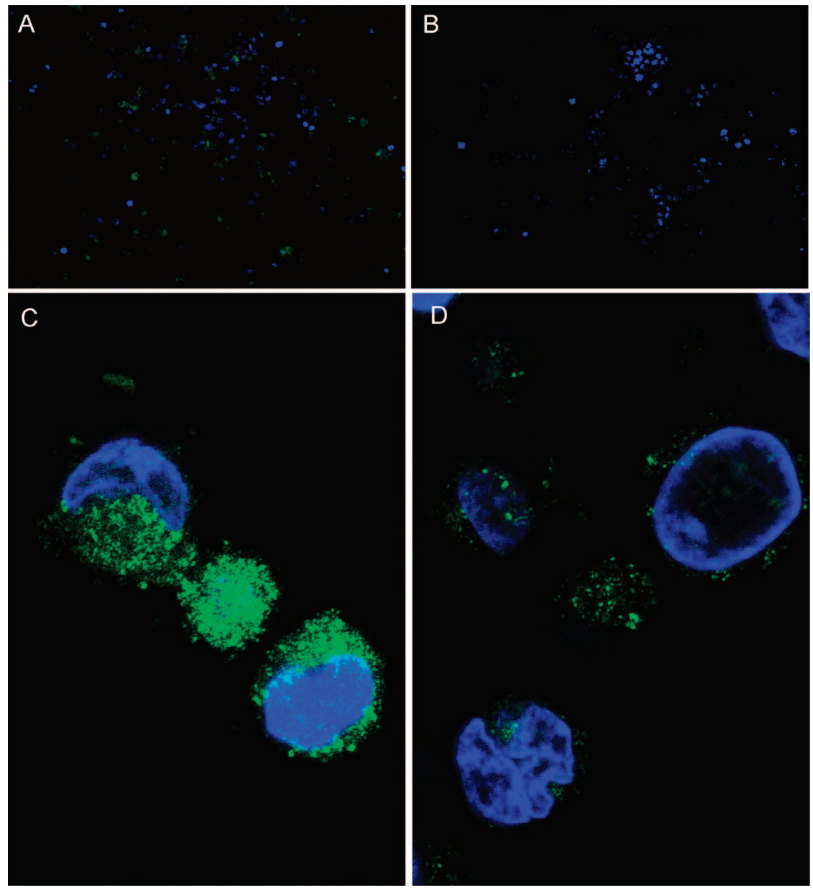

Figure 4. Nanometer-sized enzyme bioreactors (nanoreactors) based on functionalized polymer vesicles loaded with trypsin are functional in cells (A,C). Hydrophobic substrate BZiPAR is converted to green fluorescent Rhodamine 110 upon incubation with THP-1 cells containing nanoreactors loaded with trypsin but not with THP-1 cells containing empty fPVs (B,D).

stabilization.[16–18] Indeed, upon cellular uptake, nanoreactors maintain structural stability for a relatively long time, protecting encapsulated enzymes from external conditions and allowing for a prolonged use in their intended capacity.

The membrane of the polymer vesicles is selectively permeable, allowing the crossing of substrate and product molecules but preventing the release of catalytic components. Thus, trypsin, captured within nanoreactor compartments, was able to process its substrate molecules.

The results described in this work improve our understanding of the cellular uptake and trafficking of polymer nanoreactors and represent a step forward toward realizing biomedical applications of artificial subcellular nanosystems with biological functionalities. We demonstrate that polymer nanoreactors possess certain chemical and physical characteristics that are essential for a functional artificial organelle, including encapsulation of catalytic components, stable membranes, and boundaries that allow for selective diffusion of substrate molecules and products. In conclusion, we show here for the first time that nanometer-sized vesicles combined with biological components introduced to target cells can serve as specific functional artificial organelles. In the future, various combinations of polymers, ligands, transmembrane proteins, receptors, and enzymes may increase the potential of this nanosystem. Such systems could target cells involved in various diseases where they would reside for extended periods of time and function to modify cellular function of cells involved in diseases such as cancer and atherosclerosis, offering a novel paradigm for medical therapy and, beyond that, for equipping specific cells with novel designer functionality.

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