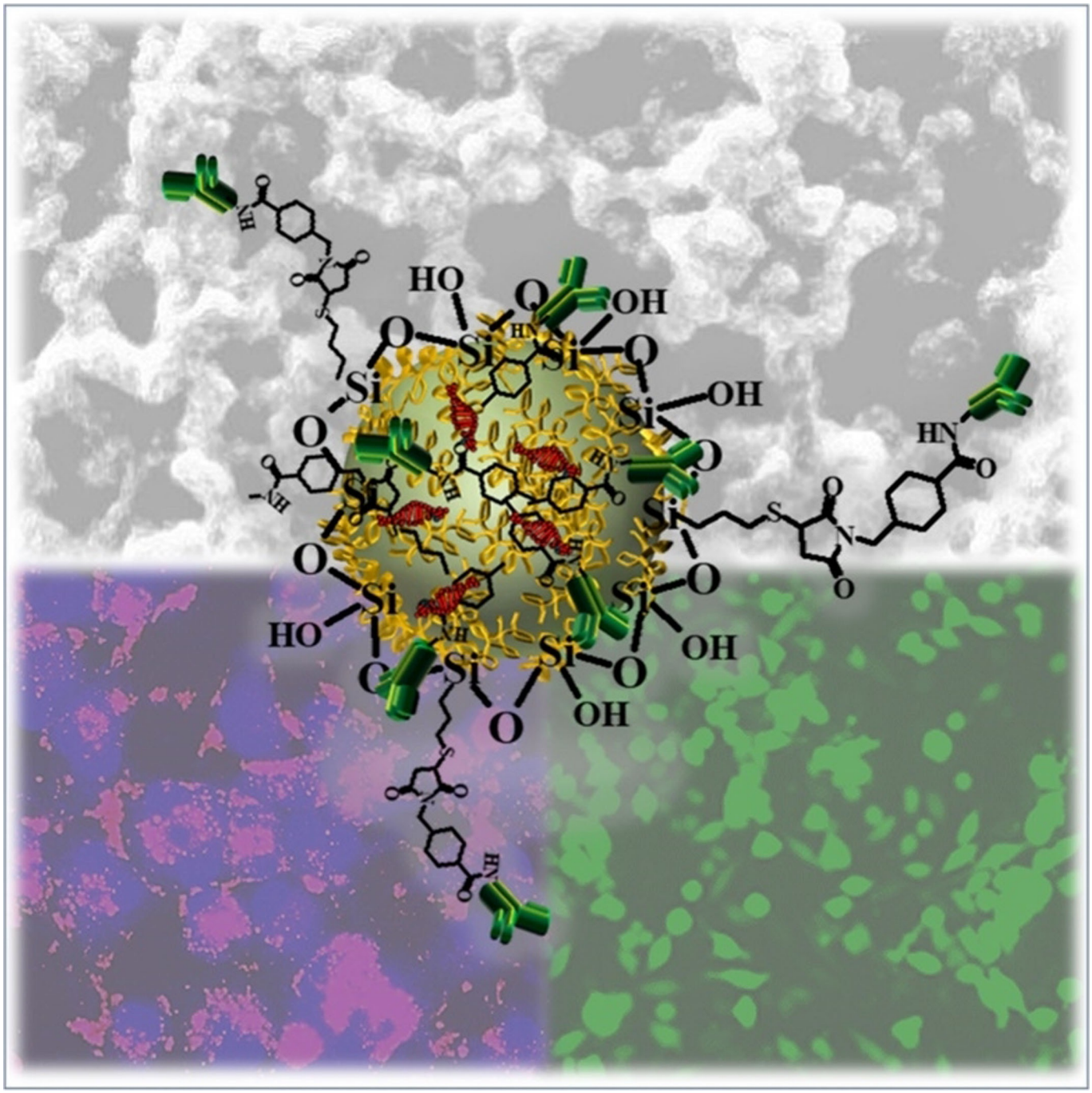
Chemistry–A European Journal
Review
doi.org/10.1002/chem.202005257
Chemistry Europe
European Chemical Societies Publishing

Nanomedicine

Biological and Medical Applications of Calcium Phosphate Nanoparticles

Viktoriya Sokolova and Matthias Epple*[a]



Abstract: Calcium phosphate nanoparticles have a high biocompatibility and biodegradability due to their chemical similarity to human hard tissue, for example, bone and teeth. They can be used as efficient carriers for different kinds of biomolecules such as nucleic acids, proteins, peptides, antibodies, or drugs, which alone are not able to enter cells where their biological effect is required. They can be loaded with cargo molecules by incorporating them, unlike solid nanoparticles, and also by surface functionalization. This offers protection, for example, against nucleases, and the possibility for cell targeting. If such nanoparticles are functionalized with fluorescing dyes, they can be applied for imaging in vitro and in vivo. Synthesis, functionalization and cell uptake mechanisms of calcium phosphate nanoparticles are discussed together with applications in transfection, gene silencing, imaging, immunization, and bone substitution. Biodistribution data of calcium phosphate nanoparticles in vivo are reviewed.

Introduction

Nanoparticles have a high surface-to-volume ratio which causes their specific physicochemical, biological, optical, electrical, and catalytic properties.[1] In general, he application of nanoparticles in biology and medicine is a rapidly growing field, for example, the efficient targeted delivery of drugs and biomolecules in vitro and in vivo in cancer therapy and immunology. Besides polymeric nanoparticles and biological nanoparticles, inorganic nanoparticles like iron oxides,[2] silica,[3] gold,[4] and calcium phosphate[5] have gained high attention due to their mechanical stability and integrity, ease of preparation, tunable size, and versatile surface chemistry. Among inorganic nanoparticles, calcium phosphate nanoparticles have distinct advantages, mainly their high biocompatibility and biodegradability. In bulk form or as coating, calcium phosphate is a well-known biomaterial which in nanoparticulate form has found many applications in vitro and in vivo.[6] Within the organism, calcium phosphate is present in biomineralized hard tissue, usually as nanoplatelets embedded in a softer protein matrix (collagen). There, it is the mineral component of bones and teeth, usually as calcium-deficient hydroxyapatite with different ionic substitutions.[6b,7] Unlike many artificial nanoparticle materials like iron oxide, polymers, silica or nanodiamonds, calcium phosphate is almost ubiquitous in the body due to its presence in bone, teeth, saliva, and blood, leading to a high biocompatibility and an intrinsic non-toxicity.[8] The main interest in calcium phosphate lies in the manufacture of biomedical implants and the elucidation of biomineralization processes,[9] but applications in molecular biology and biomedicine have also been intensively explored. In particular, calcium phosphate nanoparticles have been extensively investigated for biomedical applications in the last decades.[8]

In contrast to many other kinds of inorganic nanoparticles (e.g. gold, magnetite, silica, some polymers), calcium phosphate nanoparticles are readily soluble at the low pH inside endolysosomes or phagosomes, that is, after cellular uptake, but stable at neutral pH, for example, in the blood. They can therefore be considered as well biodegradable, in contrast to biopersistent materials like gold, nanodiamonds, magnetite, or carbon nanotubes. Furthermore, calcium phosphate nanoparticles meet many important requirements for an efficient delivery system, that is, the ability to incorporate drugs or biomolecules both inside and on the surface, either physically or covalently bound, the ability to retain such biomolecules until the particle has reached the target site and is dissolved, and its inherent biodegradation to harmless compounds (calcium and phosphate ions).[10] Polymeric nanoparticles are also very versatile, but it has to be emphasized that they are often not biodegradable and with a few exceptions, consist of monomers which are unknown to the body. Consequently, most (but not all) are not very "biomimetic" and not resorbed by the body, but simply excreted. Exceptions are biopolymers and polyesters which degrade to naturally occurring oligomers and monomers like sugars or lactic acid. Mesoporous silica nanoparticles are another example of multifunctional porous nanoparticles for drug delivery.[11] Figure 1 summarizes the main advantages of calcium phosphate nanoparticles.

Here we summarize the procedures established so far for the synthesis of calcium phosphate nanoparticles and their application in biology and medicine. First, we introduce different synthesis and characterization methods for calcium phosphate nanoparticles. Then, we discuss how calcium phosphate nanoparticles can be loaded and functionalized on the surface with different drugs and biomolecules. Finally, we review the application of functionalized calcium phosphate nanoparticles in vitro and in vivo.

Synthesis of Calcium Phosphate Nanoparticles

Different methods for the synthesis of calcium phosphate nanoparticles with different size, morphology, and composition were developed.[12] These methods are focused on the synthesis of calcium phosphate particles in the nanoscale dimension

[a] *Dr. V. Sokolova, Prof. Dr. M. Epple*
Inorganic chemistry
University of Duisburg-Essen
Universitaetsstr. 5–7, 45117 Essen (Germany)
E-mail: matthias.epple@uni-due.de

The ORCID identification number(s) for the author(s) of this article can be found under:
https://doi.org/10.1002/chem.202005257.

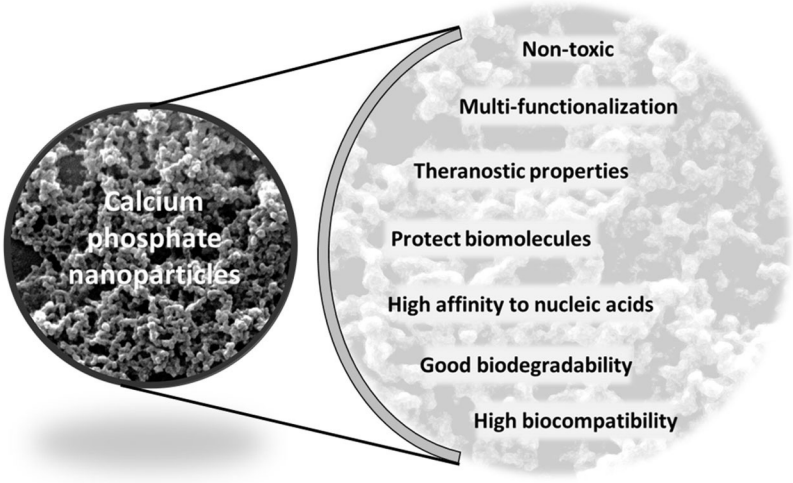


Figure 1. Advantages of calcium phosphate nanoparticles as a carrier system for biomedical applications.

(i.e., less than 100 nm) in more or less agglomerated form, for applications in biology and medicine.[13]

In general, calcium phosphate is synthesized by various methods like wet-chemical precipitation,[14] sol-gel chemistry,[15] flame spray pyrolysis,[12a,16] and solid-state reactions (Table 1). Calcium phosphate is sparingly soluble in neutral water, that is, it tends to precipitate easily from supersaturated solutions. The precipitation from water is easy, cost-efficient, and environmentally friendly, as no organic solvent is required.[17] It has some advantages like the possibility to control particle crystallinity and size by varying pH, concentration, temperature, and precipitation time.[15c,18] If performed quickly at ambient temperature, the precipitation usually leads to poorly crystalline particles.[12a,15c] A higher temperature and a lower crystallization rate enhance particle crystallinity and lead to a better defined particle shape, especially in hydrothermal processes.[19] It is possible to add (bio)organic compounds during the synthesis, unlike as in high-temperature processes or syntheses involving organic solvents where a denaturation of biomolecules like nucleic acids or proteins may occur.

The sol–gel synthesis is based on the reaction of a calcium source and a phosphate source, usually in an organic solvent.[20] It offers different possibilities to fabricate a wide range of structured nanomaterials, including coatings on metallic implants.[20a,21] The sol–gel synthesis is advantageous due to its simplicity, high versatility, comparatively homogeneous product composition (e.g. Ca:P ratio), and low synthesis temperature.[20b,22] It has been applied to prepared nanoparticles with different composition, including fluoroapatite.[23]

Flame-spray pyrolysis is a versatile method for the large-scale synthesis of calcium phosphate nanoparticles. A solution or a dispersion of the precursors is injected into a flame where the particle formation occurs at high temperature.[16a,b,17] The possibilities for precursor selection and reactor system engineering make this method suitable to produce particles with variable properties, also for biomedical applications.[12a,16a,d,17,24] The method is adjustable with respect to particle morphology, crystallinity, and size. The applied solvents or precursors, the local temperature, and the residence time in the flame all influence the combustion reaction, giving some control over primary particle size and crystalline phase, but irreversible particle agglomeration is frequently observed.[16b] Although it requires specialized equipment, flame-spray pyrolysis is an efficient method to prepare larger quantities of nanoparticulate calcium phosphate with defined properties, but the high processing temperature prevents the incorporation of all organic or biological compounds.

Pulsed laser ablation has also been applied to prepare calcium phosphate nanoparticles from synthetic[25] and biological

Viktoriya Sokolova obtained a PhD degree in Inorganic Chemistry at the University of Duisburg-Essen in the group of Prof. Epple in 2006. She received the Young Scientist Award of the Klee Family of the German Society for Biomedical Technology and the Award of the German Society for Biomaterials. Since 2011 she leads the biological research in the group of Prof. Epple at the University of Duisburg-Essen. The focus of her research is the synthesis of inorganic nanoparticles and their application in 2D and 3D cell culture models.

Matthias Epple studied chemistry at the University of Braunschweig and obtained his PhD with Prof. H.K. Cammenga in 1992. After postdoctoral studies at the University of Washington (Seattle; Prof. J.C. Berg) and the Royal Institution (London, UK; Prof. J.M. Thomas), he joined the group of Prof. A. Reller at the University of Hamburg for his habilitation. In 2000, he became Associate Professor at the University of Bochum and in 2003 Full Professor at the University of Duisburg-Essen. His research interests cover biomaterials, biomineralization, nanoscience and nanomedicine.

Table 1. Synthetic methods to prepare calcium phosphate nanoparticles.

Method	Advantages	Limitations	References
Precipitation from aqueous solutions	Bulk synthesis possible; low cost; incorporation of organic or biological compounds possible; only water as solvent	Upscaling can be difficult and requires a continuous process	[30]
Sol-gel method	Low cost; control over chemical composition	Upscaling can be difficult; organic solvents required	[31]
Flame-spray pyrolysis	Good crystallinity; possibility for scale-up	Particle agglomeration; no incorporation of organic molecules possible; special equipment necessary	[16b,c,17,24c,32]
Pulsed laser ablation	Control over product properties possible by adjustable laser parameters	Tendency for particle agglomeration; high-end laser equipment needed; difficult scale-up	[25–26]
Solid-state synthesis (high-temperature methods)	Easy and low cost; well-crystallized particles; high yield	Agglomerated particles; poor redispersability; application of organic compounds possible only after the synthesis	[18b,33]

calcium phosphate substrates.[26] This method is based on the ablation of nanoparticles from a solid substrate and has turned out to be very versatile to prepare metallic and ceramic nanoparticles.[27] However, its applicability to prepare calcium phosphate nanoparticles is probably limited in comparison to other methods where particle size and composition can be controlled more easily. Conventional solid-state chemical synthesis involves precursor salts like $CaCO_3$ and K_3PO_4 which are mixed, ground, and sintered at elevated temperature. This typically leads to well-crystalline products in high yield, but it is generally difficult to disperse the material in nanoparticulate form because the particles are sintered.[12a,24b] Physical methods like plasma spraying[28] and magnetron sputtering are used to coat metallic implants, often with nanocrystalline calcium phosphate phases.[21,29]

When comparing all methods for synthesis of calcium phosphate nanoparticles for biological application, a precipitation from aqueous solutions has distinct advantages compared to the other methods. It allows to load biomolecules into the particles or/and to functionalize them on the surface, leading to reproducible and uniform nanoparticles in stable colloidal dispersions. It also avoids organic solvents.

We want to stress that there is not only one calcium phosphate but a whole family of them, due to the different protonation states of the phosphate anion (PO_4^{3-}, HPO_4^{2-}, $H_2PO_4^{-}$) and the ability of calcium phosphates to substitute anions and cations by other ions.[11c,34] The most common calcium phosphate phase is hydroxyapatite, $Ca_5(PO_4)_3OH$, which is also the biomineral in mammalian bone and teeth.[35] In many reports involving calcium phosphate nanoparticles, the crystalline phase which is actually present is neither investigated nor known, and the material is simply denoted as "calcium phosphate".[8] The chemical nature of a given calcium phosphate sample can only be determined by crystallographic techniques, namely, X-ray diffraction (XRD) and electron diffraction (usually together with transmission electron microscopy; TEM). However, broad X-ray diffraction peaks and recrystallization artifacts in TEM often prevent a clear phase identification of nanoparticles. A frequently used parameter is the molar ratio of calcium to phosphate which is indicative but not characteristic for crystalline calcium phosphate phases.[34a,c,d] Ionic substitutions, multiphase samples, and amorphous phases (which are very difficult to detect in the presence of crystalline phases) often prevent a clear assignment because they can change the calcium to phosphate ratio. Thus, calcium phosphate nanomaterials often contain a mixture of different calcium phosphate phases. Given the fact that all of them are acid-soluble to calcium and phosphate ions, this does not particularly affect the biomedical application.

Characterization of Calcium Phosphate Nanoparticles

For most biological applications, calcium phosphate nanoparticles as all other nanoparticles have to be dispersed in water or biological media.[16d,36] The following considerations apply to many other kinds of nanoparticles as well. The colloidal stability of nanoparticles directly depends on their surface characteristics (charge, hydrophobicity, functional groups).[37] The main parameter for the estimation of colloidal stability in the case of electrostatic stabilization is the zeta potential: Nanoparticles with a zeta potential above +30 or below −30 mV are considered to be stable in aqueous dispersion, as the surface charge prevents aggregation of the particles. For instance, by applying a cationic polymer like polyethylenimine (PEI) for the stabilization of calcium phosphate nanoparticles, positively charged nanoparticles (+30 mV) are obtained. For negatively charged calcium phosphate nanoparticles (−30 mV), an anionic polymer carboxymethylcellulose (CMC) can be used. A colloidal stabilization can also be caused by the adsorption of proteins from the biological dispersion medium (e.g. blood), the so-called protein corona.[38] For the characterization of nanoparticulate systems in colloidal dispersion, different methods are generally applied. The main methods are summarized in the Table 2 with special emphasis on calcium phosphate nanoparticles. However, most considerations apply to other kinds of nanoparticles as well.

In general, different methods are usually applied for the characterization of calcium phosphate nanoparticles, as it is generally recommended for nanoparticles.[13c,48] This is imperative if nanoparticles are acting as carriers for drugs or biomolecules where the particle composition must be exactly known. In that case, regulatory bodies are demanding an in-depth characterization, for example, for biomedical or nanomedical application. At minimum, the loading with a drug or a biomolecule must be known, accompanied by particle properties like

Table 2. Possibilities and limitations of different analytical methods for the characterization of calcium phosphate nanoparticles. Scanning electron microscopy (**SEM**), transmission electron microscopy (**TEM**), dynamic light scattering (**DLS**), disc centrifugal sedimentation (**DCS**), analytical ultracentrifugation (**AUC**), nanoparticle tracking analysis (**NTA**), optical spectroscopy (**UV-VIS**), X-ray powder diffraction (**XRD**, also sometimes denoted as wide-angle X-ray scattering, **WAXS**), small-angle X-ray scattering (**SAXS**), inductively-coupled plasma—mass spectrometry (**ICP-MS**), and atomic absorption spectroscopy (**AAS**).

Method	Possibilities	Limitations	References
SEM and TEM	Size and shape of nanoparticles (only calcium phosphate core); phase identification if combined with electron diffraction (ED) and/or energy-dispersive X-ray spectroscopy (EDX) (important for calcium phosphates due to different possible phases)	Imaging in the dry state; only a small number of particles can be analyzed; possible contamination by other compounds (e.g. salts or biomolecules from the dispersion medium); beam damage may cause artifacts, especially with hydrated or amorphous calcium phosphate phases	[39]
DLS	Easy and fast determination of particle size and surface charge (zeta potential)	Works well for monodisperse systems and poorly for polydisperse systems	[5a, 40]
DCS	Easy determination of particle size	Works well for calcium phosphate nanoparticles due to their density (about 3,000 kg m^{-3})	[41]
AUC	Discrimination between particles of different size and density	Works well for calcium phosphate nanoparticles due to their density	[42]
NTA	Direct determination of particle size (hydrodynamic radius); direct visualization of dispersed particles	Analysis of only a small number of particles; large particles may sediment and remain undetected	[43]
UV-VIS	Determination of the loading with drugs and biomolecules	Fluorescent labeling of analytes is usually necessary	[44]
XRD	Identification of crystalline calcium phosphate phases; determination of nanoparticle crystallinity and domain size; detection of crystalline impurities	Only for crystalline particles; broad diffraction peaks; amorphous phases may go undetected besides crystalline phases	[45]
SAXS	Determination of hydrodynamic radii and agglomeration state; calcium phosphate is well suited due to its density	Complex data analysis	[46]
ICP-MS	Determination of overall particle composition with high precision	Analysis of single particles possible if not too big; calcium phosphate nanoparticles are usually too large (40 nm or more)	[47]
AAS	Determination of metal content, for example, calcium or substituting ions	Lower sensitivity than ICP-MS; not possible for phosphate	[44]

size, charge, and shape.[13c] In the following, we will restrict ourselves to the peculiarities of calcium phosphate nanoparticles, but most considerations can be applied to other kinds of nanoparticles as well. We will also use the overall expression "cargo" for all kinds of drug molecules or biomolecules, irrespective of their chemical nature.

Besides electron microscopy, dynamic light scattering (DLS) is probably the most prominent method to analyze the size and the surface charge (zeta potential) of dispersed calcium phosphate nanoparticles. DLS is a fast and appropriate method if the particle size distribution is monomodal and narrow. This has to be confirmed by other techniques like electron microscopy or disc centrifugation. In polydisperse systems, DLS tends to produce artifacts due to the fact that large particles scatter the light much more intensely than smaller particles (r^6 dependency of the scattering power).[41,49] If the nanoparticles are not spherical or occur as a polydisperse mixture, this will lead to false results. These may go unnoticed unless additional methods like SEM or DCS are applied to characterize the nanoparticles. Figure 2 shows typical characterization data for biomolecule-loaded calcium phosphate nanoparticles. Note that SEM probes the solid calcium phosphate core in the dry state whereas DLS probes the hydrodynamic diameter in the dispersed state, including possible aggregates of smaller primary particles.

If prepared for biomedical use, the calcium phosphate nanoparticles must be thoroughly purified to remove excess reagents from the synthesis and unwanted synthesis by-products like the inorganic counter ions of calcium phosphate. Otherwise, these will render the interpretation of biological results impossible (just imagine the effect of excess cargo molecules which are present in solution and not on the nanoparticle). Commonly applied purification techniques are centrifugation, nanofiltration, and dialysis.[44] Centrifugation is the best option for calcium phosphate nanoparticles due to their density and comparatively large diameter (typically 50 to 100 nm). After purification and size characterization, the amount of cargo must be quantitatively determined. This is more difficult than generally assumed and not always done (or reported) in the literature. Autofluorescent or fluorescently labelled drug molecules can be easily detected by UV-spectroscopy or (less accurately) by fluorescence spectroscopy.[51] This can be performed either with the cargo-loaded nanoparticles or with the supernatant that remains after nanoparticle purification. If the cargo itself is not fluorescent, a quantification is often difficult or

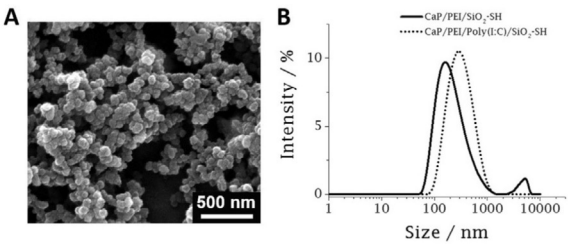


Figure 2. Scanning electron micrograph of calcium phosphate/PEI nanoparticles with an outer silica shell, loaded with the toll-like receptor ligand poly(I:C) (a nucleic acid) (**A**). Dynamic light scattering data of unloaded nanoparticles (CaP/PEI/SiO_2-SH) and of nanoparticles carrying poly(I:C) (CaP/PEI/poly(I:C)/SiO_2-SH) (**B**).[50] Reprinted from Ref. [50] with permission from Elsevier.

even impossible with standard laboratory methods (HPLC is an option after acidic dissolution of the nanoparticles). In the case of fluorescently labelled cargo molecules like proteins or antibodies, it is usually tacitly assumed that they have the same biological and physico-chemical properties as their non-labelled parent compounds. If cargo molecules are available only in small amounts (like many biomolecules), a full characterization is sometimes impossible due to the lack of material. In that case, model cargo molecules (like unspecific IgG antibodies) can be used instead of the real cargo molecules (like specific antibodies).

The number concentration of calcium phosphate nanoparticles in a dispersion can be determined by elemental analysis. Typically, AAS and ICP-MS are the methods of choice. From the calcium and/or the phosphate concentration, the concentration of calcium phosphate in the dispersion can be derived if its stoichiometry is known. Usually, the stoichiometry of the most common calcium phosphate phase hydroxyapatite, ($Ca_5(PO_4)_3OH$), is tentatively applied. Furthermore, the particle density of the calcium phosphate (hydroxyapatite: 3,140 $kg\,m^{-3}$), the particle shape (usually spherical), and a monodisperse particle size distribution must be assumed. As not all parameters are exactly known, the particle concentration in a dispersion is accessible only with limited accuracy (error at least $\pm 25\,\%$, concerning particle number concentrations).[5e,39b,52] To our experience, direct methods that claim to be able to determine absolute particle concentrations like NTA or DLS are even less accurate.

Loading and Surface Functionalization of Calcium Phosphate Nanoparticles

The efficient action of calcium phosphate nanoparticles in biological systems depends both on the core composition as well as on the surface properties.[53] Different methods for nanoparticle loading and surface functionalization were published, some of them based on physical interactions and others on the formation of covalent bonds.[48c,54] Calcium phosphate nanoparticles can be surface-functionalized with polyelectrolytes or charged macromolecules like polyethylenimine (PEI) or DNA for colloidal stabilization. This is a purely adsorptive binding between charged macromolecules and calcium phosphate particles that prevents their agglomeration and subsequent sedimentation. It is also possible to add another layer of opposite charge on top, like negatively charged DNA onto positively charged CaP/PEI, as in the layer-by-layer approach.[55] By this way, polyelectrolyte nanocapsules are accessible after acidic dissolution of the calcium phosphate core.[56] It is often better to include the cargo molecules into the nanoparticles to protect them against external degradation. This is especially important for nucleic acids like DNA and RNA which are sensitive to nucleases and RNAses. Such a protection is possible by adding another layer of calcium phosphate, followed by an outermost charged layer (e.g. PEI or DNA) for colloidal stabilization.[18a,43]

Targeting or labelling molecules can be attached or adsorbed to the surface of a calcium phosphate nanoparticle to modify the specific affinity of the material (usually for cell targeting).[4b,57] For example, antibodies, peptides, and aptamers have been applied as targeting agents by surface functionalization of different kinds of nanoparticles.[4a,39a,58] If a stable surface functionalization is desired, a purely adsorptive binding is not the method of choice. For a mere adsorption, for example, of an antibody, it is never clear how stable the attachment is under biological conditions. A better method is the covalent attachment of a molecule to the outermost adsorbed macromolecule layer (like a PEI-antibody conjugation), but this may compromise the colloidal stability and does not fully eliminate the question of adsorptive stability.

Calcium phosphate itself is an ionic compound that cannot be covalently functionalized. However, a promising approach is the attachment of a thin silica shell onto the calcium phosphate surface, followed by well-established siloxane chemistry to bind triethoxysilanes like APTES to silanol groups.[39a,59] With such an amine- or thiol-terminated nanoparticle surface, further conjugation strategies can be employed. For instance, proteins and antibodies can be attached to the nanoparticle surface. Then we have a stable covalent bond, consisting of the sequence SiO_3-*O*-C_xH_y-linker-protein. Such a covalent approach is typical for gold or silica nanoparticles where biomolecules are strongly attached on the surface.[60] However, compared to gold or silica nanoparticles, the advantage of calcium phosphate nanoparticles is the possibility to load cargo molecules into the particles. Figure 3 summaries the possible ways for preparation of functionalized calcium phosphate nanoparticles. Adsorptive and covalent binding approaches can be combined for calcium phosphate nanoparticles, leading to multifunctional nanoparticles as described above.

Using such functionalization strategies, calcium phosphate nanoparticles have been used as carriers for different biomolecules, for example, peptides for immunization,[61] proteins for imaging,[5d,62] and siRNA for gene silencing and plasmid DNA for transfection (Table 3).[63]

As outlined above, a covalent surface modification provides a more stable binding than an adsorptive physical interaction.[68] The functionalization of silica-terminated calcium phosphate nanoparticles with (3-aminopropyl)triethoxysilane (APTES) or (3-mercaptopropyl)trimethoxysilane (MPS) to introduce either amine or thiol terminal groups to the surface has been reported.[39a] Such amine terminal groups can be used to couple dyes or biomolecules by suitable crosslinkers. For instance, calcium phosphate nanoparticles have been modified on the surface with anti-CD11c antibodies to target dendritic cells,[39a] Hen Egg Lysozyme (HEL) for immunization (B-cell targeting),[5b,69] and synthetic antigens for HSV-1 (Herpes simplex virus 1).[70] A drawback of this method is the need to develop and optimize individual surface functionalization strategies for each case.

A more flexible surface conjugation is based on the non-covalent, but very strong avidin-biotin-interaction. A surface conjugation of calcium phosphate nanoparticles with avidin permits the non-covalent attachment of any biotinylated molecule. This was shown with anti-CD11c antibodies where cell targeting was demonstrated in vitro and in vivo.[58g] As another

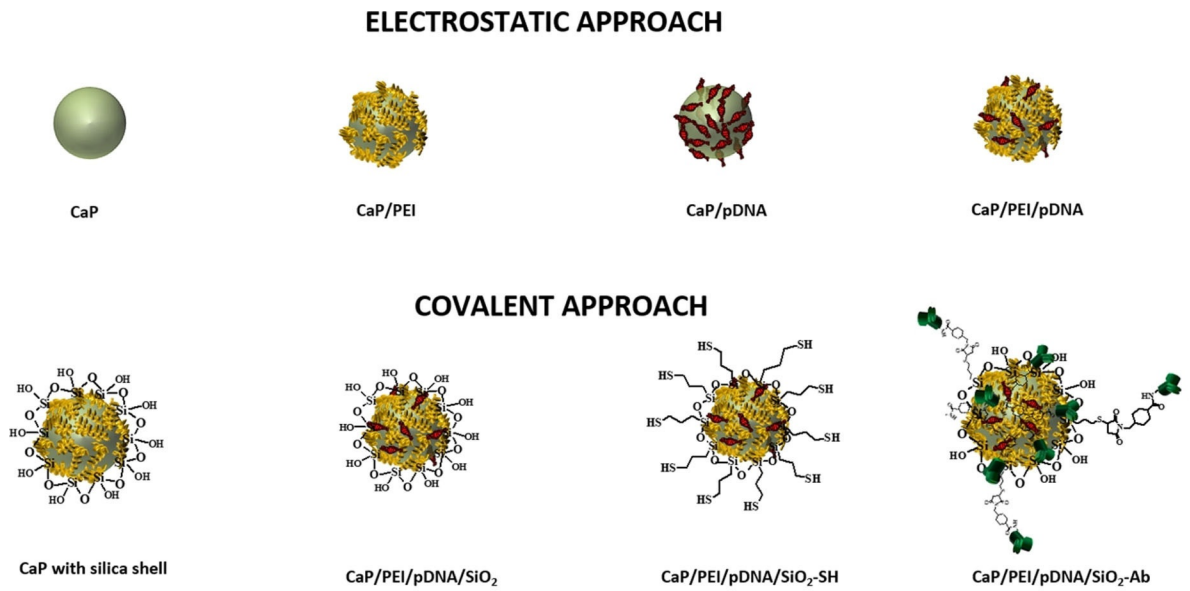


Figure 3. Principles of internal loading and surface-functionalization of calcium phosphate (CaP) nanoparticles with biomolecules (here: Plasmid DNA; pDNA). In the electrostatic approach (top row), the calcium phosphate core is coated either with the cationic polyelectrolyte PEI or with negatively charged pDNA. This leads to an electrosteric stabilization with either positive (CaP/PEI) or negative (CaP/pDNA) particle charge. It is also possible to attach another layer of pDNA onto a positively charged CaP/PEI nanoparticle. A covalent attachment of ligands to calcium phosphate is not directly possible because calcium phosphate is an ionic compound. The covalent approach is depicted in the bottom row. The coating with a silica shell (SiO_2) encapsulates PEI and pDNA and permits to conjugate molecules like antibodies (Ab) to the nanoparticle surface, using siloxane chemistry and established bioconjugation procedures.

Table 3. Loading of calcium phosphate nanoparticles with biomolecules for biological application.

Method	Cargo molecules	Examples	Application	References
Adsorptive loading	Nucleic acids (e.g. plasmid DNA, siRNA)	pDNA-EGFP pDNA-BMP7 pDNA-VEGF pDNA-HBsAg	Transfection, bone regeneration, genetic vaccination	[5e,f,64]
		siRNA-EGFP, siRNA-TNF-α	Gene silencing, anti-inflammation	[63a,65]
Covalent surface functionalization	Antibodies, peptides	DEC205, anti-Cd11c, IgG, RGDfK	Cell targeting	[39a,58g]
Loading or surface functionalization	Peptides and proteins	Antigens, BSA, lysozyme, HIV-Env, Avidin	Immunization, cell targeting	[61a,66]
Adsorption of dye-labelled polymers or covalent surface conjugation of dye molecules via click-chemistry	Dyes	Cy5, FITC, TRITC, TAMRA	Imaging, photodynamic therapy (PDT)	[39b,40b,67]

versatile surface conjugation method, the highly specific and orthogonal copper-catalyzed azide–alkyne cycloaddition (CuAAC) as most prominent click reaction[71] is possible on the nanoparticle surface. After surface-functionalization with azide, all kinds of alkyne-carrying ligands can be clicked onto the calcium phosphate surface, including dyes and proteins. A strain-promoted azide-alkyne cycloaddition (SPAAC) is as also possible (Figure 4).[39b,67]

If the particle concentration in a dispersion has been determined, it can be related to the number concentration of the cargo molecules in the dispersion. Of course, one has to ensure that all free cargo molecules have been removed by previous purification steps. Then, the number of molecules per calcium phosphate nanoparticle can be calculated.[72] This can also be related to the specific particle surface if the particle geometry (e.g. spherical) is known, giving the number of molecules per nanoparticle surface area (molecular footprint). If the cargo molecules are located inside the nanoparticle and not on its surface, the calculation is the same, but of course does not lead to the surface coverage. Table 4 gives typical data for calcium phosphate nanoparticles, loaded with different cargo molecules.

Uptake of Calcium Phosphate Nanoparticles by Cells In Vitro

An important property of calcium phosphate nanoparticles (shared with other nanoparticles) is their ability to act as transporter of specific cargo molecules into cells.[62] Depending on nanoparticle size, charge and surface functionalization, different uptake mechanisms by the cell can occur, with endocytosis and phagocytosis being the most prominent.[37a,48a,74] Smaller particles (< 200 nm) are usually taken up via endocytosis[75] which can be divided in clathrin-mediated, caveolin-mediated

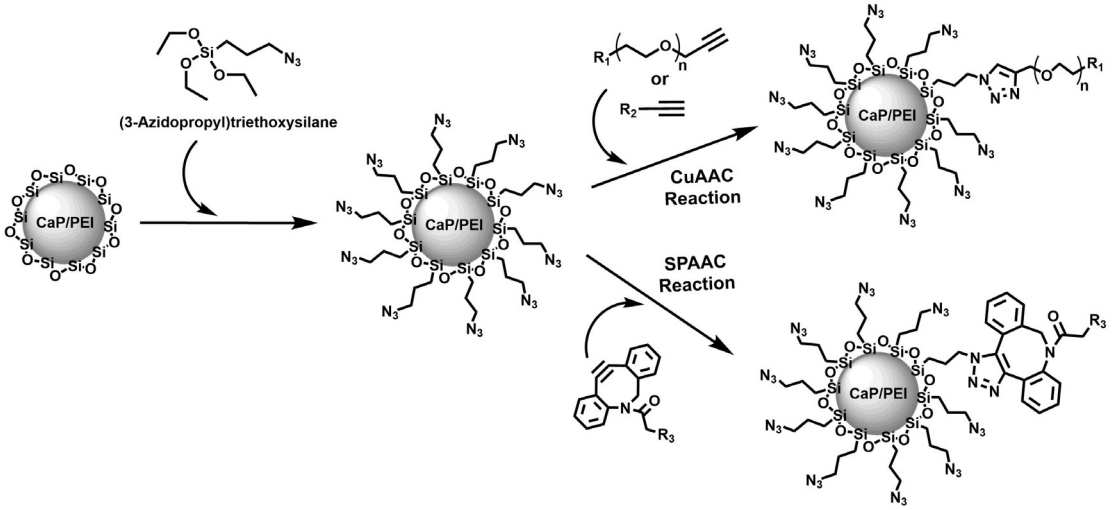


Figure 4. Two pathways for the synthesis of azide-terminated calcium phosphate nanoparticles, followed by a covalent surface modification either by CuAAC (copper-catalyzed azide–alkyne cycloaddition) or SPAAC (strain-promoted azide–alkyne cycloaddition) click chemistry.[39b,67] Reprinted with permission from Ref. [39b]. Copyright 2020, Wiley-VCH.

Table 4. Loading of calcium phosphate nanoparticles with different cargo molecules. (**pDNA-EGFP**: Plasmid encoding for enhanced green fluorescent protein; **Poly(I:C)**: Double-stranded RNA-analogue polyinosinic-polycytidylic acid, a Toll-like receptor 3 ligand; **siRNA-TNF-α**: anti-tumor necrosis factor alpha (TNF-α) siRNA. **Tandem**: EGFP-mRFP1 fusion protein.

Nanoparticle type and diameter	Cargo molecule	Location of cargo molecule	Method of cargo fixation	Number of cargo molecules per nanoparticle	Surface density of cargo molecule/nm^2	Reference
CaP/PEI/Poly(I:C)/SiO_2-SH (120 nm)	Poly(I:C) (130 kDa)	Inside	Adsorption	900	–	[5f]
CaP/PEI/pDNA-EGFP/SiO_2 (64 nm)	pDNA-EGFP (4056 kDa)	Inside	Adsorption	131	–	[5f]
CaP/PEI/siRNA-TNF-α/SiO_2 (40 nm)	siRNA-TNF-α (M = 3,460 g mol^{-1})	Inside	Adsorption	2,040	–	[65c]
CaP/PEI/SiO_2-Env (40 nm)	HIV Env-trimer (140 kDa)	Surface	Covalent attachment	865	0.17	[66b]
CaP/PEI/Tandem/CaP/PEI (131 nm)	EGFP-mRFP-Tandem protein (52 kDa)	Inside	Adsorption	7,100	–	[73]
CaP/PEI/SiO_2-S-Avidin (120 nm)	Avidin (62 kDa)	Surface	Covalent attachment	240	0.03	[58g]
CaP/PEI/SiO_2-FAM (62 nm)	FAM-alkyne (M = 413 Da)	Surface	Covalent attachment (CuAAc)	12,200	1.01	[39b]
CaP/PEI/SiO_2-FAM (70 nm)	FAM-alkyne (M = 413 Da)	Surface	Covalent attachment (SPAAC)	3,230	0.21	[39b]

and clathrin/caveolae-independent endocytosis. Clathrin-mediated endocytosis can occur by receptor-specific or non-specific adsorptive uptake. In the case of receptor-specific uptake, the protein clathrin mediates the process in which a clathrin-rich cavity is formed after the particle recognition, followed by an invagination of the cell membrane. With the help of adaptors and other accessory proteins, the membrane internalization is stabilized.[74b]

Caveolin-mediated endocytosis occurs via caveolae membrane invaginations with a size around 50 nm. As soon as the particle is recognized, secondary proteins help with membrane stabilization and vesicle encapsulation. Compared to the clathrin-mediated endocytosis, the caveolin-mediated endocytosis favors a lysosomal escape that prevents degradation.[75] Inhibitors permit a more or less complete shutdown of individual endocytosis pathways. The uptake mechanisms of positively and negatively charged calcium phosphate nanoparticles have been studied in vitro (Figure 5).[5d,76] It was shown that calcium phosphate nanoparticles are taken up by HeLa cells by macropinocytosis,[72] going first into early endosomes and then into endolysosomes where they are dissolved.[5d,73]

Confocal laser scanning microscopy and flow cytometry are the most common techniques to quantify the particle uptake, but both work only if the particles have been fluorescently labelled or carry a fluorescent cargo molecule.[5d,30b,62] This can also be accomplished by doping the calcium phosphate with fluorescent lanthanoids.[59,77] By high-resolution microscopy, for example, structured illuminated microscopy (SIM) and stochastic optical reconstruction microscopy (STORM) beyond the diffraction limit, individual fluorescing calcium phosphate nano-

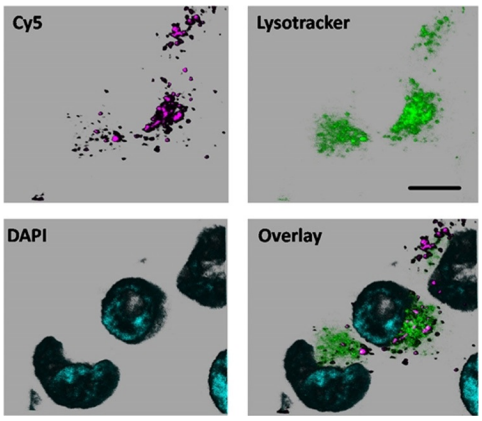


Figure 5. Uptake of red fluorescent CaP/PEI-Cy5/SiO_2 nanoparticles by HeLa cells after 24 h incubation investigated by confocal laser scanning microscopy. The calcium phosphate particles are mostly co-localized with endolysosomes. Cy5-labelled nanoparticles (magenta), lysotracker for endolysosomes (green), DAPI for cell nuclei (blue), and overlay are shown. Scale bar 10 μm.[5e] Reprinted from Ref. [5e] with permission from Elsevier.

particles were localized inside the endolysosomes of HeLa cells after uptake (Figure 6).[39b,67] Clearly, different microscopic techniques have different resolutions, but scanning electron microscopy is not directly applicable for nanoparticles which were taken up by cells. Transmission electron microscopy is also not optimal to look for calcium phosphate nanoparticles inside cells due to the low scattering contrast of calcium phosphate and the presence of various electron-rich particle-like objects inside a cell, especially after the drying for TEM preparation. Rasel et al. studied the biophysical responses of cells, that is, stiffness and adhesive properties, after the incubation with calcium phosphate nanoparticles by Raman spectroscopy. They found an increase in cell stiffness after the uptake of nanoparticles and a decrease in cell adhesion.[78]

Proteins are a major class of biomolecules which typically cannot cross the cell membrane alone, therefore a carrier to enable their transport inside the cell is needed.[38c,76,79] The endocytosis of calcium phosphate nanoparticles, loaded with the autofluorescent protein phycoerythrin, confirmed that the particles end up in endolysosomes where a degradation of the protein by nucleases occurs.[5d] Kollenda et al. presented calcium phosphate nanoparticles which were loaded with either a Tandem fusion protein or a plasmid encoding for this protein, and studied their uptake and intracellular processing by different cell lines. This Tandem protein combined two fluorescent molecules (mRFP1-EGFP) and served as biological pH sensor that allowed to track the nanoparticles and the proteins inside the cells. Time-lapse confocal microscopy confirmed that the Tandem-loaded nanoparticles were directed to endolysosomes after endocytic uptake.[73]

The uptake of calcium phosphate nanoparticles has also been studied in 3D cell culture models on spheroids generated from HeLa cells with a diameter around 500 μm. The distribution of red fluorescent calcium phosphate nanoparticles inside the spheroids was visualized by confocal laser scanning microscopy. It was also shown on the cellular level that nanoparticles were taken up by single cells inside a spheroid (Figure 7).[5e]

After cellular uptake, calcium phosphate nanoparticles end up in endolysosomes where they dissolve at the low pH (about 4) and incorporated cargo molecules are released. The influx of acid to dissolve the basic calcium phosphate probably

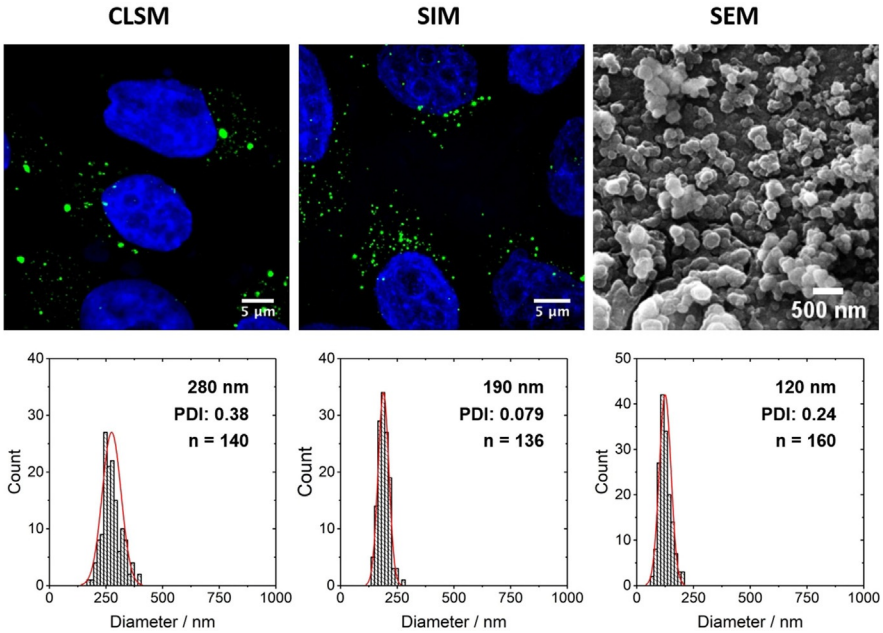


Figure 6. Uptake and particle size distribution of green-fluorescent CaP/PEI/SiO_2-Alexa488 nanoparticles by HeLa cells determined by CLSM and SIM after 24 h incubation. Pure nanoparticles without cells were investigated by SEM for comparison. Note the different resolution capabilities of these methods. However, SEM is not usually applicable for nanoparticles after uptake into cells. Nuclei were stained blue with DAPI.[39b] Reprinted with permission from Ref. [39b]. Copyright 2020, Wiley-VCH.

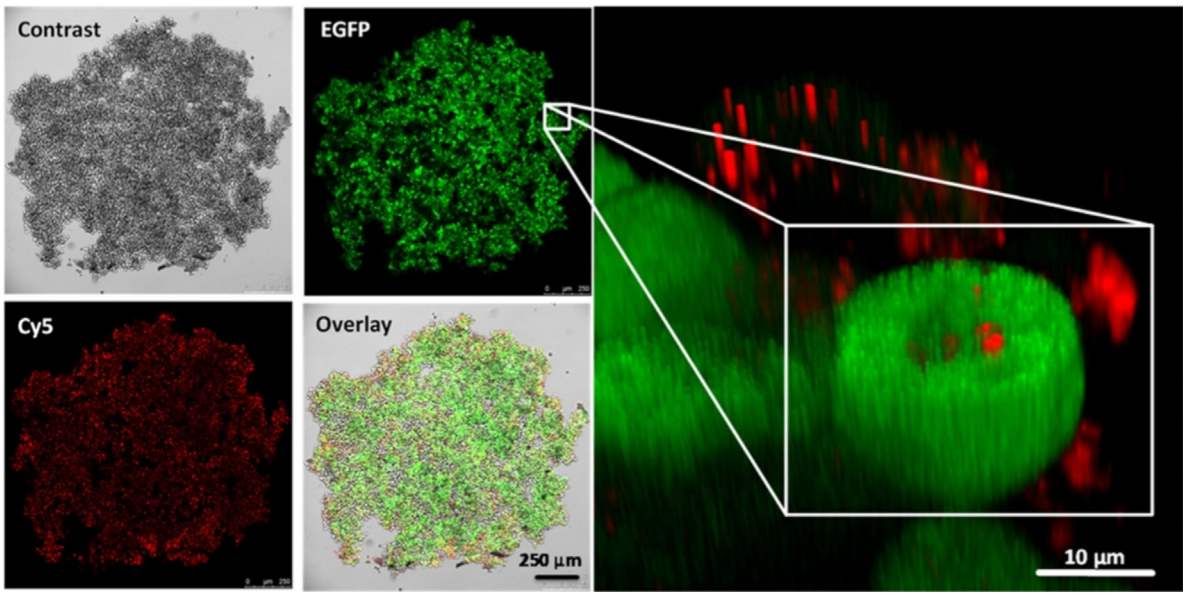


Figure 7. Representative images of green-fluorescent HeLa-EGFP spheroids incubated with red fluorescent CaP/PEI-Cy5/SiO_2 nanoparticles for 24 h by confocal laser scanning microscopy (**left**). The presence of nanoparticles inside one cell at high magnification (**right**).[5e] Reprinted from Ref. [5e] with permission from Elsevier.

leads to an increase of the osmotic pressure inside the endolysosome due to ion release which leads to its rupture and an endosomal escape.[63b,73] This is similar to the proton sponge effect reported for polyethyleneimine[80] and a clear advantage of calcium phosphate nanoparticles. This effect is important to achieve a delivery of cargo molecules into the cytosol before they suffer lysosomal degradation by low pH and degrading enzymes (e.g. proteases, nucleases). If cells are subjected to very high doses of calcium phosphate nanoparticles, an overload of calcium may occur that disrupts the intracellular calcium balance. This can even lead to cell death as cells have a very low internal calcium concentration.[81] However, under all "reasonable" conditions, calcium phosphate nanoparticles will not lead to adverse effects towards cells and organisms.[8] The released calcium ions are usually pumped out of the cell within minutes and hours.[82]

Biomedical Applications of Calcium Phosphate Nanoparticles

Unfunctionalized calcium phosphate nanoparticles have a long tradition for the treatment of bone defects. After all, bone contains calcium phosphate nanoparticles, therefore they are a natural choice as biomaterial. Calcium phosphate nanoparticles have been prepared in pasty form and injected into bone defects with good results for bone regeneration.[83] Of course, such particles are highly agglomerated due to the absence of surface functionalization, but for this application, a pasty form is highly appropriate. In contrast to sintered calcium phosphate ceramics, calcium phosphate nanoparticles are resorbed faster by osteoclasts due to their higher specific surface area and their higher solubility under acidic conditions.[84] Micro- and nanoscopic calcium phosphate (unfunctionalized) is also used in various toothpastes in order to enhance the tooth repair process. The underlying idea is to fill cavities and tubules in dentin and to remineralize enamel.[85]

Nanoparticles for therapeutic nanomedical applications are typically between 10 to 100 nm in size to ensure a good biodistribution.[86] Depending on their size and charge, the circulation time of nanoparticles in the bloodstream is variable. Nanoparticles smaller than 30 nm are rapidly excreted in the kidney by renal filtration whereas nanoparticles bigger than 200 nm are eliminated by phagocytosis.[87] It was also found that nanoparticles of 30–150 nm are found in bone marrow, heart, kidney and stomach whereas nanoparticles with a size of 150–300 nm mainly end up in the liver and in the spleen.[88] Furthermore, the cellular uptake of nanoparticles can be strongly influenced by a protein corona, which covers the particles after their contact with biological fluids.[38i,89] There is a vast literature on nanomedicine and nanomedical approaches (see, for example,[48a,90]), but here we will discuss only the applications of calcium phosphate nanoparticles. As outlined above, calcium phosphate nanoparticles can carry different cargo molecules both inside and on the surface and also more than one species in the same nanoparticle. Therefore, they are also able to simultaneously deliver different types of biomolecules, for example, a fluorophore, an antigen, and an adjuvant for immunization, to one designated cell or tissue.

The introduction of foreign plasmid DNA into eukaryotic cells is called transfection. After successful entry of desired plasmid DNA into the nucleus, the expression of the gene and the following production of specific protein occurs.[5c,65c,91] As DNA alone is not able to cross the cell membrane and also prone to enzymatic degradation, a suitable delivery system is required. Different kinds of nanoparticles, dendrimers, and liposomes have been developed for transfection, including calcium

phosphate nanoparticles.[36c,92] The standard calcium phosphate transfection method was introduced by Graham and van der Eb in 1973.[93] This method is based on a co-precipitation of calcium phosphate and DNA, leading to poorly defined nano- and microparticulate aggregates. Better defined calcium phosphate nanoparticles are therefore promising candidates as delivery vehicles for DNA, also due to their good biocompatibility and high biodegradability.[63c,91,94]

DNA-loaded calcium phosphate nanoparticles are usually prepared by a precipitation of calcium phosphate from calcium- and phosphate-containing solutions, followed by a rapid colloidal stabilization with polyelectrolytes, including nucleic acids.[5e,40a,43,64c,82,95] The advantage of this controlled preparation in comparison to the conventional in situ precipitation method is a much better control over size, charge, and composition of these particles.[5c,e,96] For a successful transfection, the particle properties (e.g. size and charge), the loading with DNA, and the particle concentration in the medium play decisive roles.[91,94d,97] As protective coating, another shell of calcium phosphate,[18a] a silica shell,[39a] or an outer polymer layer (e.g. polylactide) can be applied.[31d] It has been demonstrated that the incorporation of DNA into the nanoparticle enhances the transfection efficiency compared to an outer shell of DNA, obviously due to a better protection of DNA from nucleases. Chernousova et al. compared the transfection efficiency of calcium phosphate nanoparticles and Lipofectamine (a liposomal transfection agent) by live-cell imaging (Figure 8).[63c] The absolute number of transfected cells by calcium phosphate nanoparticles was comparable to Lipofectamine, but the advantage of calcium phosphate was its lower cytotoxicity in contrast to Lipofectamine, which is a major criterion for a potential in vivo application.[63c]

As ceramic, putty or paste, calcium phosphate is clinically used for bone regeneration in orthopedic surgery and dentistry.[34e,98] In these areas, calcium phosphate nanoparticles are especially useful for the delivery of plasmid DNA to induce the production of specific proteins for bone healing or vascularization. Tenkumo et al. applied plasmid DNA-loaded calcium phosphate nanoparticles in collagen scaffolds for gene transfection in vivo. They induced the production and a prolonged release of bone morphogenetic protein-2 (BMP-2) after successful transfection from these scaffolds.[64c] Khalifehzadeh et al. developed a single-step synthesis method to prepare Sr-doped calcium phosphate nanoparticles with a diameter of about 300 nm. They showed that the cellular uptake increased with decreasing strontium content. The presence of strontium in the nanoparticles had a strong influence on the gene transfection efficiency and the alkaline phosphatase activity in a human fetal osteoblastic cell line.[99] Krebs et al. reported an in vivo gene delivery system based on alginate hydrogels containing calcium phosphate nanoparticles loaded with DNA with a size around 100 nm, encoding for BMP-2.[100] Hadjicharalambous et al. reported that calcium phosphate nanoparticles loaded with plasmid DNA encoding for BMP-7 induced an osteogenic response in pre-osteoblasts after transfection, which was demonstrated by measuring the alkaline phosphatase (ALP) activity and calcium phosphate deposition with alizarin red staining.[64b] Keneey et al. prepared collagen/calcium phosphate scaffolds which were loaded with a plasmid encoding vascular endothelial growth factor$_{165}$ (pVEGF$_{165}$) and served as a delivery system. They showed a successful transfection from such scaffolds, indicating that no additional transfection vectors were needed.[64a] A bioactive paste based on DNA-loaded calcium phosphate nanoparticles for the in situ transfection of BMP-7 and VEGF-A was investigated in vitro[94b] and also in vivo. This bioactive paste led to a faster initial healing of a critical-size bone defect in the rabbit tibia.[94c] Rojas et al. reported that plasmid DNA-loaded calcium phosphate nanoparticles activated antigen-presenting cells better than the free biomolecules and that the transfection efficiency of such nanoparticles was comparable with the efficiency of the plasmid alone applied by electroporation.[5f]

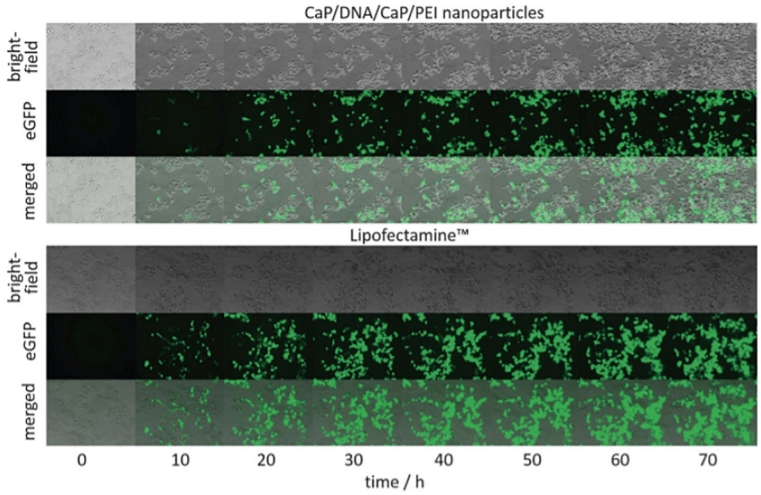


Figure 8. Live-cell fluorescence microscopy imaging of HeLa cells during transfection with EGFP-pDNA-loaded nanoparticles and Lipofectamine. The transfection efficiency (indicated by green fluorescent cells) increases with increasing incubation time.[63c] Reprinted with permission from Ref. [63c]. Copyright 2017, Springer Nature.

Genetic disorders can also be treated by gene silencing techniques, that is, RNA interference (RNAi), which is based on the specific inhibition of the protein synthesis in a cell.[6a,101] On the cellular level, the synthesis of a protein is inhibited via double-stranded RNA (dsRNA) which has to be transported inside the cell. After entering the cell, dsRNA is processed by an endonuclease into small interfering RNA (siRNA) which is complementary to messenger RNA (mRNA) of the specific protein. Finally, after multiple processes in the cell, the biosynthesis of the protein is inhibited. The main challenge for a therapeutic application of gene silencing is the delivery of siRNA into the target cells, as siRNA alone only to small extent permeates the cell membrane.[39c] Furthermore, siRNA is highly unstable in the body as it is degraded by RNAses.[102] There has been intensive work on the delivery of RNA for a therapeutic application, including vaccination, usually by nanoparticles, liposomes, and dendrimers, following the same pathways as with transfection.[92d,103]

Calcium phosphate nanoparticles are able to successfully deliver and release siRNA inside a cell. Different strategies have been developed to load calcium phosphate nanoparticles with siRNA, usually by adsorption onto cationic nanoparticles or by incorporation during synthesis.[63a,65b,101c,104] Devarasu et al. carried out investigations with hybrid siRNA-loaded calcium phosphate nanoparticles, demonstrating a high gene silencing efficiency (up to 95%) in luciferase-expressing cells in vitro and their biodistribution in mice, with their localization mainly in the liver.[105] Pittella et al. described calcium phosphate/polymer hybrid nanoparticles, loaded with VEGF-silencing siRNA, and showed a high gene silencing efficiency in vitro in pancreatic cancer cells and a significant reduction in the subcutaneous tumor growth, together with VEGF gene silencing in the tumor.[106] Tobin et al. developed siRNA/doxorubicin-loaded PEGylated multi-shell calcium phosphate nanoparticles to induce gene silencing of XIAP (X-linked inhibitor of apoptosis protein) in a tumor in vivo.[107] Wang et al. reported siRNA-loaded calcium phosphate nanospheres which effectively induced cell apoptosis in vitro.[108] Gene silencing of inflammatory cytokines is a promising treatment for chronic inflammations. Frede et al. reported the application of calcium phosphate nanoparticles loaded with siRNA directed against pro-inflammatory cytokines (e.g. TNF-α, IFN-γ, IP-10 and CCL-2) for specific gene silencing in vivo.[63a,65b] Tenkumo et al. developed a bioactive calcium phosphate paste containing siRNA against TNF-α. Its anti-inflammatory effects were shown in vitro and in vivo in a rat periodontitis model.[65c]

Calcium phosphate nanoparticles have also been successfully applied for vaccination and immunization, for example, to induce a specific host immunity against infectious diseases.[44,109] They can serve as vaccine delivery system that allows the flexible dosage of adjuvants and antigens in one nanoparticle, which is important for the optimization of vaccines.[52a] Calcium phosphate nanoparticles have been loaded with the oligonucleotides CpG (TLR9 ligand; an adjuvant) and hemagglutinin (a short peptide, a viral antigen from the influenza A virus) and applied in vitro and in vivo in mice. It is important that the different cargo molecules are present in and on one and the same nanoparticle, which is difficult or impossible to achieve with solid nanoparticles like gold or iron oxide. The protection offered by calcium phosphate nanoparticles for cargo packaged inside is a clear advantage. The nanoparticles were efficiently taken up by dendritic cells in vivo and induced a strong immune response in immunized mice.[44,109d,110] Furthermore, calcium phosphate nanoparticles, functionalized with CpG and retroviral T cell epitopes (Friend virus) were efficient against acute and chronic retroviral infections. The immunization of Friend virus-infected mice reactivated effector T-cells and led to a significant decrease in viral loads (Figure 9).[61,66a] Sahdev et al. developed ovalbumin-sugar-coated calcium phosphate nanoparticles and applied them in mice by intradermal injection. It was shown that the production of ovalbumin-specific antibodies in vivo was strongly induced.[111] Biomolecules like CpG, poly(I:C) or flagellin which serve as adjuvants during immunization can also be loaded into calcium phosphate nanoparticles, thereby enhancing the immune response.[69,112] Lin et al. reported that biocompatible calcium phosphate nanoparticles with tunable characteristics can also act as adjuvants by inducing more balanced Th1 and Th2 immune responses.[109b] Chiu et al. synthesized protein-coated calcium phosphate nanoparticles with a size of 100 nm and applied them as adjuvant/antigen delivery system to dendritic cells.[113] Kopp et al. developed calcium phosphate nanoparticles which were internally loaded with CpG and surface-coated with a synthetic peptide specific for herpes simplex virus type 1 (HSV-1). The immunization of mice with such nanoparticles resulted in the production of cell-to-cell spread-inhibiting antibodies.[70] Morcol et al. showed that the application of the recombinant hemagglutinin protein by calcium phosphate nanoparticles resulted in a significantly higher IgG production, hemagglutination inhibition, and decreased virus titers in mice.[114] Temchura et al. reported calcium phosphate nanoparticles which were loaded with adjuvants and surface-functionalized with the protein antigen HEL, resulting in antigen-specific targeting and primary activation of naïve B-cells in vivo.[5b,69] Damm et al. prepared calcium phosphate nanoparticles loaded with p30 (T-helper epitope of Tetanus toxoid), functionalized the surface with Env (protein trimers of the HIV-1 envelope) and applied them for immunization in a mouse model. These nanoparticles induced the activation of naïve Env-specific B-cells in vitro and were able to induce HIV-1 Env-specific antibody responses in vivo.[66b] In most of these cases, the multifunctionality of the calcium phosphate nanoparticles was of decisive influence, that is, that all cargo molecules were transported into the same cell with one nanoparticle.

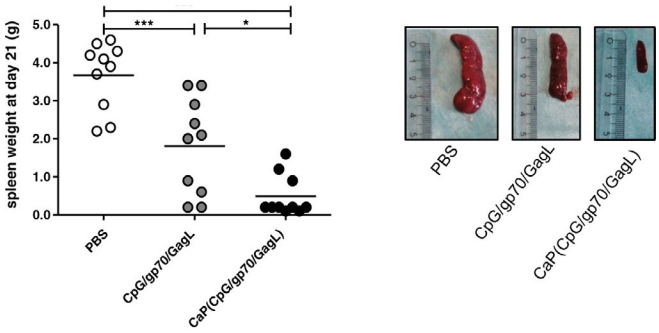


Figure 9. Immunization of mice with functionalized calcium phosphate nanoparticles protected them from the Friend virus (FV)-induced splenomegaly (pathologic spleen growth) and reduced the viral loads. **Left:** Spleen weight at day 21 post-infection. **Right:** Representative images of a spleen per group at day 21 post-infection. PBS: Control; CpG/gp70/GagL: Mixture of dissolved biomolecules; CaP(CpG/gp70/GagL): Three different biomolecules, loaded to the same calcium phosphate nanoparticles.[66a] Reprinted from Ref. [66a] with permission from Elsevier.

Instead of carrying the required protein, it is also possible to deliver the plasmid of interest that encodes the require protein into the cell to induce an immune response (genetic vaccination). In such an approach, calcium phosphate nanoparticles were loaded with plasmid DNA encoding for the hepatitis B virus surface antigen (HBSAg) and applied for transfection in vitro and in vivo.[39b]

Tissue engineering is a promising tool for regenerative medicine, but the search for suitable scaffolds composed of a bioac-

tive degradable substrate is still a challenge.[86,115] In general, calcium phosphate nanoparticles are well suited in tissue engineering for hard tissue regeneration (mainly bone) due to their presence in bone, causing their biocompatibility, biodegradability, bioactivity, osteoconductivity, and osteoinductivity. Scaffolds for hard tissue regeneration can be supplemented by bioactive calcium phosphate nanoparticles that are loaded with stimulating biomolecules. For instance, it is possible to stimulate tissue growth by releasing proteins or DNA for transfection, loaded to calcium phosphate nanoparticles.[86,116] It is also possible to enhance the mechanical properties of organic scaffolds by the mineral calcium phosphate.[117] Accordingly, calcium phosphate has been incorporated into different natural and synthetic polymers to produce nanocomposites with specific mechanical and biomedical properties.[118] Chitosan, collagen, gelatin, polycaprolactone, and poly(lactic acid) are examples of polymers which are widely used as biodegradable matrix for such nanocomposites.[119]

In Vivo Tracing of Calcium Phosphate Nanoparticles

In vivo effects of nanoparticles depend on many parameters, for example, the particle size, the agglomeration tendency, the circulation time in the body, the immunogenicity, the cellular uptake, the intracellular trafficking, the degradation by cells, the physicochemical dissolution, the flow properties in the bloodstream and the clearance from the blood.[48a,120] Optical microscopy in the near-infrared (NIR) region is a valuable tool to follow suitably labelled nanoparticles in vivo. Antinoglu et al. have synthesized and studied bioresorbable calcium phosphate nanoparticles carrying the near-infrared emitting fluorophore indocyanine-green. Nanoparticles accumulated in solid xenograft breast adenocarcinoma tumors (5 mm diameter) within 24 h after systemic tail vein injection in a nude mouse model.[31b] Figure 10 shows fluorescence distributions of Cy5-siRNA-loaded nanoparticles and of free dissolved Cy5-siRNA in SMMC-7721 tumor-bearing mice 4 h and 24 h after injection. Cy5-siRNA nanoparticles accumulated in tumors within 4 h and were observed up to 24 h after injection at the tumor site in contrast to free Cy5-siRNA which was mostly eliminated 4 h post-injection. Furthermore, no fluorescence signals of free Cy5-siRNA were observed in the tumor or major organs 24 h post-injection. However, significant fluorescence signals were observed in the liver and the lung after injection of Cy5-siRNA loaded nanoparticles.[101b] Haedicke et al. prepared fluorescently labelled calcium phosphate nanoparticles (DY682; NIR dye) and followed their pathway in mice after tail-vein injection. After 24 h, the nanoparticles were mainly found in the lung and in the liver.[5f,40b,50]

Optical methods are restricted by the absorption in tissue, even in the NIR region. Therefore, other methods must be applied if a look deeper into the body is required. Positron-emission tomography (PET) is an imaging technique that uses radioactive isotopes to visualize and measure metabolic activities of cells and to trace radioisotope-labelled molecules and nanoparticles in vivo.[121] Labelling with ^{18}F is a good option for calcium phosphate nanoparticles because fluoride is readily incorporated to form insoluble fluorapatite, $Ca_5(PO_4)_3F$. Jauregui-Osoro et al. reported that hydroxyapatite nanoparticles bind [^{18}F]-fluoride in various biological media. The in vivo behavior of [^{18}F]-labelled hydroxyapatite nanoparticles was studied by PET-CT imaging in mice.[122] Kollenda et al. functionalized calcium phosphate nanoparticles with the metal ligand DOTA by covalent surface-coupling and labeled them with the PET radioisotope ^{68}Ga.[52b] The biodistribution of these nanoparticles was investigated by small animal PET-CT. After intravenous injection into mice, the particles were mobile and traveled with the bloodstream mostly into liver and lung (Figure 11).

Figure 12 shows the ex vivo distribution of CaP-PEI-SiO_2-S-DOTA-^{68}Ga nanoparticles 5 h post-injection. Most nanoparticles were found in spleen, lung and liver after intravenous injection. After intramuscular, intratumoral, and soft-tissue injection, the particles predominantly stayed at the injection site during the observation period.[52b] Lobaz et al. followed intravenously administered radiolabeled polymer-coated hydroxyapatite nanoparticles by single-photon emission computed tomography (SPECT) visualization.[123] Maia et al. synthesized ^{99m}Tc-radiolabeled mesoporous hydroxyapatite nanoparticles and studied their biodistribution in mice. A high concentration was observed in liver and spleen after uptake by macrophages.[124]

The intravenous injection of calcium phosphate nanoparticles leads to a pronounced distribution in the body, with liver, lung and spleen the most prominent target organs. Their long-term fate is unknown, but based on the known cell-biological effects, it can be safely assumed that they will be taken up by

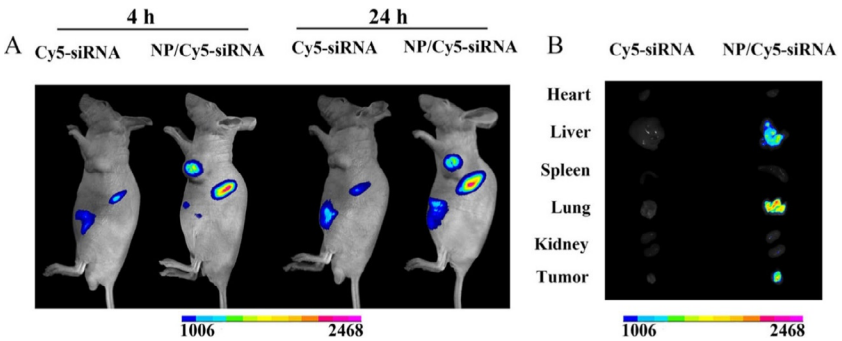


Figure 10. In vivo NIR fluorescence imaging of Cy5-siRNA loaded nanoparticles and free Cy5-siRNA in SMMC-7721 tumor-bearing mice at 4 h and 24 h post-injection (**A**). Fluorescence images of tumors and organs at 24 h post-injection (**B**).[101b] Reprinted from Ref. [101b], by Gao et al. (2016).

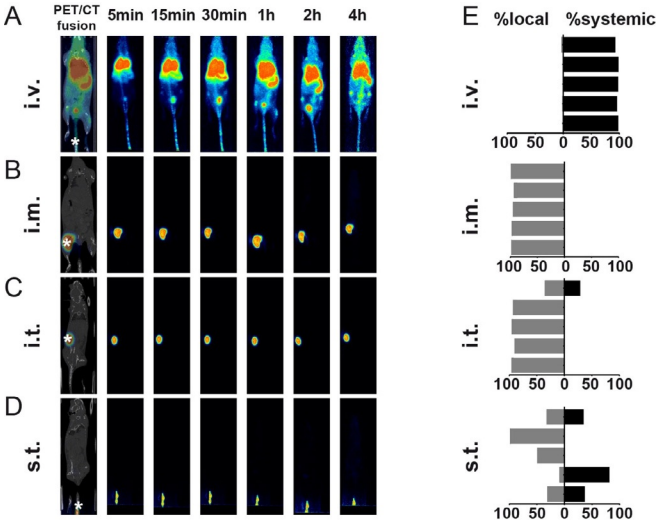


Figure 11. Representative whole-body maximum intensity projections of one BALB/c mouse for each group after injection of CaP-PEI-SiO_2-S-DOTA-^{68}Ga nanoparticles. Intravenous (*i.v.*) injection in healthy mice (**A**); intramuscular (*i.m.*) injection in healthy mice (**B**); intratumoral (*i.t.*) injection in tumor-bearing BALB/c mice (**C**); soft tissue (*s.t.*) injection in healthy mice (**D**). In vivo local and systemic biodistribution of nanoparticles for five mice per group (**E**).[52b] * Injection site. Reprinted from Ref. [52b] with permission from Elsevier.

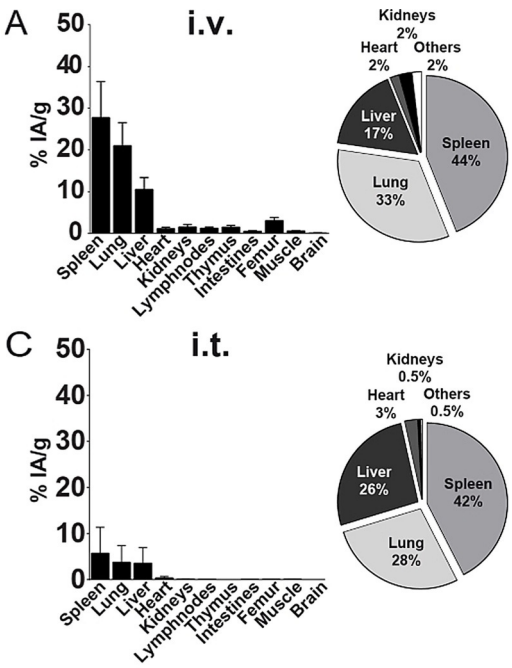


Figure 12. Ex vivo biodistribution of CaP-PEI-SiO_2-S-DOTA-^{68}Ga nanoparticles in healthy and tumor-bearing BALB/c mice by gamma counter measurement of extracted organs after sacrifice 5 h post-injection. Intravenous (*i.v.*) injection in healthy mice (**A**); intratumoral (*i.t.*) injection in tumor-bearing BALB/c mice. Most nanoparticles remained in the tumor, and only a small fraction was mobile in the body as detailed in the pie diagram (**C**).[52b] Reprinted from Ref. [52b] with permission from Elsevier.

cells (including macrophages) and dissolved to the constituting harmless ions, that is, Ca^{2+} and PO_4^{3-}, unlike other insoluble inorganic and polymeric nanoparticles. Upon direct injection into tissue or a tumor, the calcium phosphate nanoparticles are much less mobile which represents an attractive option for a local drug delivery, induced by locally injected nanoparticles. The question whether a targeted delivery by nanoparticles is possible is controversially discussed in nanomedicine for all kinds of nanoparticles and beyond the focus of this review.[48a,90b,125]

In summary, not only the composition, but also the size of nanoparticles plays a critical role in their biodistribution in the body. Calcium phosphate nanoparticles are usually of the order of 100 nm and therefore end up in lung, liver or spleen where they are taken up by cells and degraded to harmless ions.[8]

Summary and Outlook

Among inorganic materials, calcium phosphate nanoparticles are well suited to transport organic or biological molecules into cells and tissue. They are well tolerated by cells and organisms and have a clearly defined degradation pathway, also due to their almost ubiquitous presence in the body. If they are used as carriers, they are advantageous to encapsulate sensitive cargo like proteins or nucleic acids inside to protect them from enzymatic degradation, something that is impossible with solid nanoparticles. A surface functionalization further enhances their potential for a targeted delivery, for example, to tumors or cells of the immune system. Polymeric or liposomal particles are competing strategies, but it is advantageous that calcium phosphate nanoparticles contain the same components as biomineralized mammalian tissue (unlike synthetic polymers) and that they have a solid core with high mechanical stability (unlike liposomes). Therefore, we expect more widespread applications in different areas of cell biology and medicine in the future as the basic synthetic steps have been explored and several in vivo studies have demonstrated their potential. Promising applications are seen in drug and gene delivery and also in immunization, for example, for vaccination.

Acknowledgements

We thank the Deutsche Forschungsgemeinschaft (DFG) for funding in the framework of the Collaborative Research Center SFB/CRC 1093: Supramolecular Chemistry on Proteins. Open access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest.

Keywords: calcium phosphate · drug delivery · gene therapy · nanomedicine · nanoparticles

[1] a) S. E. Lohse, C. J. Murphy, *J. Am. Chem. Soc.* **2012**, *134*, 15607 – 15620; b) S. Naz, M. Shamoon, R. Wang, L. Zhang, J. Zhou, J. Chen, *Int. J. Mol. Sci.* **2019**, *20*, 965.

[2] a) K. E. Albinali, M. M. Zagho, Y. Deng, A. A. Elzatahry, *Int. J. Nanomedicine* **2019**, *Volume 14*, 1707–1723; b) A. K. Hauser, M. I. Mitov, E. F. Daley, R. C. McGarry, K. W. Anderson, J. Z. Hilt, *Biomaterials* **2016**, *105*, 127–135.
[3] a) R. Roggers, S. Kanvinde, S. Boonsith, D. Oupický, *AAPS PharmSciTech* **2014**, *15*, 1163–1171; b) S. Kesse, K. O. Boakye-Yiadom, B. O. Ochete, Y. Opoku-Damoah, F. Akhtar, M. S. Filli, M. A. Farooq, M. Aquib, B. J. M. Mily, G. Murtaza, B. Wang, *Pharmaceutics* **2019**, *11*, 77.
[4] a) P. Singh, S. Pandit, V. R. Mokkapati, A. Garg, V. Ravikumar, I. Mijakovic, *Int. J. Mol. Sci.* **2018**, *19*, 1979; b) V. Sokolova, G. Nzou, S. B. van der Meer, T. Ruks, M. Heggen, K. Loza, N. Hagemann, F. Murke, B. Giebel, D. M. Hermann, A. J. Atala, M. Epple, *Acta Biomater.* **2020**, *111*, 349–362.
[5] a) R. Ramachandran, W. Paul, C. P. Sharma, *J. Biomed. Mater. Res. Part B: Appl. Biomater. Sect. B* **2009**, *88*, 41–48; b) V. Temchura, D. Kozlova, V. Sokolova, K. Überla, M. Epple, *Biomaterials* **2014**, *35*, 6098–6105; c) B. Mostaghaci, B. Loretz, C. M. Lehr, *Curr. Pharm. Des.* **2016**, *22*, 1529–1533; d) M. Kopp, O. Rotan, C. Papadopoulos, N. Schulze, H. Meyer, M. Epple, *PLOS One* **2017**, *12*, e0178260; e) V. Sokolova, L. Rojas-Sanchez, N. Bialas, N. Schulze, M. Epple, *Acta Biomater.* **2019**, *84*, 391–401; f) L. Rojas-Sánchez, E. Zhang, V. Sokolova, M. Zhong, H. Yan, M. Lu, L. Li, H. Yan, M. Epple, *Acta Biomater.* **2020**, *110*, 254–265.
[6] a) T. J. Levingstone, S. Herbaj, J. Redmond, H. O. McCarthy, N. J. Dunne, *Nanomaterials* **2020**, *10*, 146; b) F. Ridi, I. Meazzini, B. Castroflorio, M. Bonini, D. Berti, P. Baglioni, *Adv. Colloid Interface Sci.* **2017**, *244*, 281–295.
[7] S. V. Dorozhkin, *Biomatter* **2011**, 121–164.
[8] M. Epple, *Acta Biomater.* **2018**, *77*, 1–14.
[9] N. Eliaz, N. Metoki, *Materials* **2017**, *10*, 334.
[10] S. Bose, S. Tarafder, J. Edgington, A. Bandyopadhyay, *JOM* **2011**, *63*, 93–98.
[11] a) M. Colilla, M. Vallet-Regí, *Int. J. Mol. Struct.* **2020**, *21*, 8605; b) M. Vallet-Regí, M. Colilla, I. Izquierdo-Barba, M. Manzano, *Molecules* **2017**, *23*, 47; c) D. Arcos, A. R. Boccaccini, M. Bohner, A. Diez-Perez, M. Epple, E. Gomez-Barrena, A. Herrera, J. A. Planell, L. Rodriguez-Manas, M. Vallet-Regi, *Acta Biomater.* **2014**, *10*, 1793–1805.
[12] a) M. Sadat-Shojai, M. T. Khorasani, E. Dinpanah-Khoshdargi, A. Jamshidi, *Acta Biomater.* **2013**, *9*, 7591–7621; b) K. Lin, C. Wu, J. Chang, *Acta Biomater.* **2014**, *10*, 4071–4102; c) M. Okada, T. Matsumoto, *Jap. Dent. Sci. Rev.* **2015**, *51*, 85–95; d) I. da Silva Brum, J. J. de Carvalho, J. L. da Silva Pires, M. A. A. de Carvalho, L. B. F. Dos Santos, C. N. Elias, *Sci. Rep.* **2019**, *9*, 19602.
[13] a) D. R. Boverhof, C. M. Bramante, J. H. Butala, S. F. Clancy, M. Lafranconi, J. West, S. C. Gordon, *Regul. Toxicol. Pharmacol.* **2015**, *73*, 137–150; b) M. Boholm, R. Arvidsson, *NanoEthics* **2016**, *10*, 25–40; c) M. Miernicki, T. Hofmann, I. Eisenberger, F. von der Kammer, A. Praetorius, *Nat. Nanotechnol.* **2019**, *14*, 208–216.
[14] a) Z. Z. Zyman, D. V. Rokhmistrov, V. Glushko, *J. Mater. Sci. Mater. Med.* **2009**, *20*, 1389; b) T. de Araujo, S. Souza, W. Miyakawa, M. Sousa, *Mater. Chem. Phys.* **2010**, *124*, 1071–1076.
[15] a) R. Jahandideh, A. Behnamghader, M. Rangie, A. Youzbashi, S. Joughehdoust, R. Tolouei, *Key Eng. Mater.* **2009**, *396*, 607–610; b) A. Hanifi, M. H. Fathi, H. Mir Mohammad Sadeghi, J. Varshosaz, *J. Mater. Sci. Mater. Med.* **2010**, *21*, 2393–2401; c) T. Welzel, W. Meyer-Zaika, M. Epple, *Chem. Commun.* **2004**, 1204–1205.
[16] a) S. L. Loher, W. J. Stark, M. Maciejewski, A. Baiker, S. E. Pratsinis, D. Reichardt, F. Maspero, F. Krumeich, D. Günther, *Chem. Mater.* **2005**, *17*, 36–42; b) W. Y. Teoh, R. Amal, L. Mädler, *Nanoscale* **2010**, *2*, 1324–1347; c) S. Ataol, A. Tezcaner, O. Duygulu, D. Keskin, N. E. Machin, *J. Nanopart. Res.* **2015**, *17*, 95; d) W. J. Stark, *Angew. Chem. Int. Ed.* **2011**, *50*, 1242–1258; *Angew. Chem.* **2011**, *123*, 1276–1293.
[17] D. Mohn, N. Doebelin, S. Tadier, R. E. Bernabei, N. A. Luechinger, W. J. Stark, M. Bohner, *J. Mater. Chem.* **2011**, *21*, 13963.
[18] a) V. V. Sokolova, I. Radtke, R. Heumann, M. Epple, *Biomaterials* **2006**, *27*, 3147–3153; b) M. Ebrahimi, M. Botelho, W. Lu, N. Monmaturapoj, *J. Biomed. Mater. Res. A* **2019**, *107*, 1654–1666.
[19] a) I. S. Neira, Y. V. Kolenko, O. I. Lebedev, G. Van Tendeloo, H. S. Gupta, F. Guitian, M. Yoshimura, *Cryst. Growth Des.* **2009**, *9*, 466–474; b) S. Kuśnieruk, J. Wojnarowicz, A. Chodara, T. Chudoba, S. Gierlotka, W. Lojkowski, *Beilstein J. Nanotechnol.* **2016**, *7*, 1586–1601.
[20] a) G. J. Owens, R. K. Singh, F. Foroutan, M. Alqaysi, C. M. Han, C. Mahapatra, H. W. Kim, J. C. Knowles, *Prog. Mater. Sci.* **2016**, *77*, 1–79; b) K. P. Sanosh, M. C. Chu, A. Balakrishnan, T. N. Kim, S. J. Cho, *Curr. Appl. Phys.* **2010**, *10*, 68; c) R. Gupta, A. Kumar, *Biomed. Mater.* **2008**, *3*, 034005; d) H. Q. Nguyen, D. A. Deporter, R. M. Pilliar, N. Valiquette, R. Yakubovich, *Biomaterials* **2004**, *25*, 865–876.
[21] R. A. Surmenev, M. A. Surmeneva, A. A. Ivanova, *Acta Biomater.* **2014**, *10*, 557–579.
[22] a) J. Wang, L. L. Shaw, *J. Mater. Sci. Mater. Med.* **2009**, *20*, 1223–1227; b) S. Bose, S. Tarafder, *Acta Biomater.* **2012**, *8*, 1401–1421.
[23] R. G. Simões, A. I. Aleixo, A. L. C. Lagoa, M. E. Minas da Piedade, J. P. Leal, T. Peitsch, M. Epple, *J. Therm. Anal. Calorim.* **2010**, *100*, 509–517.
[24] a) A. R. Boccaccini, M. Erol, W. J. Stark, D. Mohn, Z. Hong, J. F. Mano, *Compos. Sci. Technol.* **2010**, *70*, 1764–1776; b) H. Zhou, J. Lee, *Acta Biomater.* **2011**, *7*, 2769–2781; c) N. Hild, R. Fuhrer, D. Mohn, S. B. Bubenhofer, R. N. Grass, N. A. Luechinger, K. Feldman, C. Dora, W. J. Stark, *Biomed Mater.* **2012**, *7*, 054103.
[25] M. Boutinguiza, J. Pou, F. Lusquiños, R. Comesaña, A. Riveiro, *Phys. Proc.* **2011**, *12*, 54–59.
[26] M. Boutinguiza, R. Comesaña, F. Lusquiños, A. Riveiro, J. Pou, *Nanoscale Res. Lett.* **2011**, *6*, 255–255.
[27] a) S. Reichenberger, G. Marzun, M. Muhler, S. Barcikowski, *ChemCatChem* **2019**, *11*, 4489–4518; b) D. Zhang, B. Gökce, S. Barcikowski, *Chem. Rev.* **2017**, *117*, 3990–4103.
[28] R. B. Heimann, *Surf. Coat. Technol.* **2006**, *201*, 2012–2019.
[29] a) A. A. Ivanova, M. A. Surmeneva, R. A. Surmenev, D. Depla, *Thin Solid Films* **2015**, *591*, 368–374; b) R. A. Surmenev, M. A. Surmeneva, I. Y. Grubova, R. V. Chernozem, B. Krause, T. Baumbach, K. Loza, M. Epple, *Appl. Surf. Sci.* **2017**, *414*, 335–344.
[30] a) X. Cheng, L. Kuhn, *Int. J. Nanomed.* **2007**, *2*, 667–674; b) K. Ganesan, A. Kovtun, S. Neumann, R. Heumann, M. Epple, *J. Mater. Chem.* **2008**, *18*, 3655–3661; c) K. Ganesan, M. Epple, *New J. Chem.* **2008**, *32*, 1326–1330; d) M. Leskiv, A. L. C. Lagoa, H. Urch, J. Schwiertz, M. E. M. da Piedade, M. Epple, *J. Phys. Chem. C* **2009**, *113*, 5478–5484; e) D. Hagmeyer, K. Ganesan, J. Ruesing, D. Schunk, C. Mayer, A. Dey, N. A. J. M. Sommerdijk, M. Epple, *J. Mater. Chem.* **2011**, *21*, 9219–9223.
[31] a) T. A. Kuriakose, S. N. Kalkura, M. Palanichamy, D. Arivuoli, K. Dierks, G. Bocelli, C. Betzel, *J. Cryst. Growth* **2004**, *263*, 517–523; b) E. I. Altınoğlu, T. J. Russin, J. M. Kaiser, B. M. Barth, P. C. Eklund, M. Kester, J. H. Adair, *ACS Nano* **2008**, *2*, 2075–2084; c) F. Bakan, O. Laçin, H. Sarac, *Powder Technol.* **2013**, *233*, 295–302; d) G. Dördelmann, D. Kozlova, S. Karczewski, R. Lizio, S. K. Knauer, M. Epple, *J. Mater. Chem. B* **2014**, *2*, 7250–7259.
[32] a) E. Boanini, M. Gazzano, A. Bigi, *Acta Biomater.* **2010**, *6*, 1882–1894; b) V. Tsikourkitoudi, J. Karlsson, P. Merkl, E. Loh, B. Henriques-Normark, G. A. Sotiriou, *Molecules* **2020**, *25*, 1747.
[33] R. L. Karlinsey, A. C. Mackey, *J. Mater. Sci.* **2009**, *44*, 346–349.
[34] a) S. V. Dorozhkin, *J. Mater. Chem. B* **2019**, *7*, 7471–7489; b) S. V. Dorozhkin, *Acta Biomater.* **2010**, *6*, 4457–4475; c) S. V. Dorozhkin, *Acta Biomater.* **2010**, *6*, 715–734; d) S. V. Dorozhkin, M. Epple, *Angew. Chem. Int. Ed.* **2002**, *41*, 3130–3146; *Angew. Chem.* **2002**, *114*, 3260–3277; e) W. Habraken, P. Habibovic, M. Epple, M. Bohner, *Mater. Today* **2016**, *19*, 69–87.
[35] J. W. C. Dunlop, P. Fratzl, *Ann. Rev. Mater. Res.* **2010**, *40*, 1–24.
[36] a) H. Goesmann, C. Feldmann, *Angew. Chem. Int. Ed.* **2010**, *49*, 1362–1395; *Angew. Chem.* **2010**, *122*, 1402–1437; b) K. Riehemann, S. W. Schneider, T. A. Luger, B. Godin, M. Ferrari, H. Fuchs, *Angew. Chem. Int. Ed.* **2009**, *48*, 872–897; *Angew. Chem.* **2009**, *121*, 886–913; c) V. Sokolova, M. Epple, *Angew. Chem. Int. Ed.* **2008**, *47*, 1382–1395; *Angew. Chem.* **2008**, *120*, 1402–1416; d) G. Schmid, W. G. Kreyling, U. Simon, *Arch. Toxicol.* **2017**, *91*, 3011–3037.
[37] a) N. Feliu, X. Sun, R. A. Alvarez Puebla, W. J. Parak, *Langmuir* **2017**, *33*, 6639–6646; b) A. Bruinink, J. Wang, P. Wick, *Arch. Toxicol.* **2015**, *89*, 659–675; c) C. Bantz, O. Koshkina, T. Lang, H. J. Galla, C. J. Kirkpatrick, R. H. Stauber, M. Maskos, *Beilstein J. Nanotechnol.* **2014**, *5*, 1774–1786.
[38] a) J. S. Gebauer, M. Malissek, S. Simon, S. K. Knauer, M. Maskos, R. H. Stauber, W. Peukert, L. Treuel, *Langmuir* **2012**, *28*, 9673–9679; b) L. Shang, G. U. Nienhaus, *Acc. Chem. Res.* **2017**, *50*, 387–395; c) M. Kopp, S. Kollenda, M. Epple, *Acc. Chem. Res.* **2017**, *50*, 1383–1390; d) P. Jain, R. S. Pawar, R. S. Pandey, J. Madan, S. Pawar, P. K. Lakshmi, M. S. Sudheesh, *Biotechnol. Adv.* **2017**, *35*, 889–904; e) D. Docter, D. Westmeier,

M. Markiewicz, S. Stolte, S. K. Knauer, R. H. Stauber, *Chem. Soc. Rev.* **2015**, *44*, 6094 – 6121; f) C. D. Walkey, W. C. W. Chan, *Chem. Soc. Rev.* **2012**, *41*, 2780 – 2799; g) S. Lara, F. Alnasser, E. Polo, D. Garry, M. C. Lo Giudice, D. R. Hristov, L. Rocks, A. Salvati, Y. Yan, K. A. Dawson, *ACS Nano* **2017**, *11*, 1884 – 1893; h) P. M. Kelly, C. Aberg, E. Polo, A. O'Connell, J. Cookman, J. Fallon, Z. Krpetic, K. A. Dawson, *Nat. Nanotechnol.* **2015**, *10*, 472 – 479; i) M. P. Monopoli, C. Åberg, A. Salvati, K. A. Dawson, *Nat. Nanotechnol.* **2012**, *7*, 779 – 786.

[39] a) D. Kozlova, S. Chernousova, T. Knuschke, J. Buer, A. M. Westendorf, M. Epple, *J. Mater. Chem.* **2012**, *22*, 396 – 404; b) L. Rojas-Sánchez, V. Sokolova, S. Riebe, J. Voskuhl, M. Epple, *ChemNanoMat* **2019**, *5*, 436 – 446; c) V. Sokolova, A. Kovtun, O. Prymak, W. Meyer-Zaika, E. A. Kubareva, E. A. Romanova, T. S. Oretskaya, R. Heumann, M. Epple, *J. Mater. Chem.* **2007**, *17*, 721 – 727.

[40] a) J. Klesing, S. Chernousova, M. Epple, *J. Mater. Chem.* **2012**, *22*, 199 – 204; b) K. Haedicke, D. Kozlova, S. Gräfe, U. Teichgräber, M. Epple, I. Hilger, *Acta Biomater.* **2015**, *14*, 197 – 207.

[41] D. Mahl, J. Diendorf, W. Meyer-Zaika, M. Epple, *Coll. Surf. A* **2011**, *377*, 386 – 392.

[42] a) D. Gebauer, A. Völkel, H. Cölfen, *Science* **2008**, *322*, 1819 – 1822; b) V. Sokolova, O. Prymak, W. Meyer-Zaika, H. Cölfen, H. Rehage, A. Shukla, M. Epple, *Mater.-wiss. u. Werkstofftech.* **2006**, *37*, 441 – 445.

[43] V. Sokolova, S. Neumann, A. Kovtun, S. Chernousova, R. Heumann, M. Epple, *J. Mater. Sci.* **2010**, *45*, 4952 – 4957.

[44] V. Sokolova, T. Knuschke, J. Buer, A. M. Westendorf, M. Epple, *Acta Biomater.* **2011**, *7*, 4029 – 4036.

[45] P. R. A. F. Garcia, K. Loza, S. Daumann, V. Grasmik, K. Pappert, A. Rostek, J. Helmlinger, O. Prymak, M. Heggen, M. Epple, C. L. P. Oliveira, *Braz. J. Phys.* **2019**, *49*, 183 – 190.

[46] a) Y. G. Sun, Y. Ren, *Part. Part. Syst. Charact.* **2013**, *30*, 399 – 419; b) C. L. P. Oliveira, S. Juul, H. L. Jorgensen, B. Knudsen, D. Tordrup, F. Oteri, M. Falconi, J. Koch, A. Desideri, J. S. Pedersen, F. F. Andersen, B. R. Knudsen, *ACS Nano* **2010**, *4*, 1367 – 1376.

[47] C. K. Su, Y. C. Sun, *J. Anal. At. Spectrom.* **2015**, *30*, 1689 – 1705.

[48] a) B. Pelaz, C. Alexiou, R. A. Alvarez-Puebla, F. Alves, A. M. Andrews, S. Ashraf, L. P. Balogh, L. Ballerini, A. Bestetti, C. Brendel, S. Bosi, M. Carril, W. C. W. Chan, C. Chen, X. Chen, X. Chen, Z. Cheng, D. Cui, J. Du, C. Dullin, A. Escudero, N. Feliu, M. Gao, M. George, Y. Gogotsi, A. Grünweller, Z. Gu, N. J. Halas, N. Hampp, R. K. Hartmann, M. C. Hersam, P. Hunziker, J. Jian, X. Jiang, P. Jungebluth, P. Kadhiresan, K. Kataoka, A. Khademhosseini, J. Kopeček, N. A. Kotov, H. F. Krug, D. S. Lee, C.-M. Lehr, K. W. Leong, X.-J. Liang, M. Ling Lim, L. M. Liz-Marzán, X. Ma, P. Macchiarini, H. Meng, H. Möhwald, P. Mulvaney, A. E. Nel, S. Nie, P. Nordlander, T. Okano, J. Oliveira, T. H. Park, R. M. Penner, M. Prato, V. Puntes, V. M. Rotello, A. Samarakoon, R. E. Schaak, Y. Shen, S. Sjöqvist, A. G. Skirtach, M. G. Soliman, M. M. Stevens, H.-W. Sung, B. Z. Tang, R. Tietze, B. N. Udugama, J. S. VanEpps, T. Weil, P. S. Weiss, I. Willner, Y. Wu, L. Yang, Z. Yue, Q. Zhang, Q. Zhang, X.-E. Zhang, Y. Zhao, X. Zhou, W. J. Parak, *ACS nano* **2017**, *11*, 2313 – 2381; b) H. F. Krug, K. Nau, *ChemBioEng Rev.* **2017**, *4*, 331 – 338; c) J. J. Giner-Casares, M. Henriksen-Lacey, M. Coronado-Puchau, L. M. Liz-Marzán, *Mater. Today* **2016**, *19*, 19 – 28.

[49] a) Y. Dieckmann, H. Colfen, H. Hofmann, A. Petri-Fink, *Anal. Chem.* **2009**, *81*, 3889 – 3895; b) A. Letzel, B. Gökce, A. Menzel, A. Plech, S. Barcikowski, *Appl. Surf. Sci.* **2018**, *435*, 743 – 751.

[50] V. Sokolova, Z. Shi, S. Huang, Y. Du, M. Kopp, A. Frede, T. Knuschke, J. Buer, D. Yang, J. Wu, A. M. Westendorf, M. Epple, *Acta Biomater.* **2017**, *64*, 401 – 410.

[51] V. Sokolova, M. Epple, *Nanoscale* **2011**, *3*, 1957 – 1962.

[52] a) F. Scheffel, T. Knuschke, L. Otto, S. Kollenda, V. Sokolova, C. Cosmovici, J. Buer, J. Timm, M. Epple, A. M. Westendorf, *Vaccines* **2020**, *8*, 110; b) S. A. Kollenda, J. Klose, T. Knuschke, V. Sokolova, J. Schmitz, M. Staniszewska, P. F. Costa, K. Herrmann, A. M. Westendorf, W. P. Fendler, M. Epple, *Acta Biomater.* **2020**, *109*, 244 – 253.

[53] Y. Cai, R. Tang, *J. Mater. Chem.* **2008**, *18*, 3775 – 3787.

[54] a) S. Kango, S. Kalia, A. Celli, J. Njuguna, Y. Habibi, R. Kumar, *Prog. Polym. Sci.* **2013**, *38*, 1232 – 1261; b) D. A. Richards, A. Maruani, V. Chudasama, *Chem. Sci.* **2017**, *8*, 63 – 77.

[55] a) G. Decher, *Science* **1997**, *277*, 1232 – 1237; b) X. Zhang, A. Kovtun, C. Mendoza-Palomares, M. Oulad-Abdelghani, S. Facca, F. Fioretti, J. C. Voegel, M. Epple, N. Benkirane-Jessel, *Biomaterials* **2010**, *31*, 6013 – 6018.

[56] a) J. Ruesing, O. Rotan, C. Gross-Heitfeld, C. Mayer, M. Epple, *J. Mater. Chem. B* **2014**, *2*, 4625 – 4630; b) J. Schwiertz, W. Meyer-Zaika, L. Ruiz-Gonzalez, J. M. Gonzales-Calbet, M. Vallet-Regi, M. Epple, *J. Mater. Chem.* **2008**, *18*, 3831 – 3834.

[57] W. R. Algar, T. Jeen, M. Massey, W. J. Peveler, J. Asselin, *Langmuir* **2019**, *35*, 7067 – 7091.

[58] a) R. T. Busch, F. Karim, J. Weis, Y. Sun, C. Zhao, E. S. Vasquez, *ACS Omega* **2019**, *4*, 15269 – 15279; b) H. Bassiony, S. Sabet, T. A. S. El-Din, M. M. Mohamed, A. A. El-Ghor, *PLoS One* **2014**, *9*, e111960; c) R. C. Popescu, E. Andronescu, B. S. Vasile, *Nanomaterials* **2019**, *9*, 1791; d) E. Jung, S. W. Kim, A. Cho, Y. J. Kim, G. J. Jeong, J. Kim, S. H. Bhang, T. Yu, *Materials* **2019**, *12*, 3850; e) R. Narayan, U. Y. Nayak, A. M. Raichur, S. Garg, *Pharmaceutics* **2018**, *10*, 118; f) R. R. Castillo, M. Vallet-Regí, *Int. J. Mol. Sci.* **2019**, *20*, 929; g) S. B. van der Meer, T. Knuschke, A. Frede, N. Schulze, A. M. Westendorf, M. Epple, *Acta Biomater.* **2017**, *57*, 414 – 425.

[59] M. Neumeier, L. A. Hails, S. A. Davis, S. Mann, M. Epple, *J. Mater. Chem.* **2011**, *21*, 1250 – 1254.

[60] a) S. A. Bansal, V. Kumar, J. Karimi, A. P. Singh, S. Kumar, *Nanoscale Adv.* **2020**, *2*, 3764 – 3787; b) R. S. Darweesh, N. M. Ayoub, S. Nazzal, *Int. J. Nanomed.* **2019**, *Volume 14*, 7643 – 7663; c) A. Graczyk, R. Pawlowska, D. Jedrzejczyk, A. Chworos, *Molecules* **2020**, *25*, 204; d) M. Homberger, U. Simon, *Phil. Trans. R. Soc. A* **2010**, *368*, 1405 – 1453.

[61] a) T. Knuschke, O. Rotan, W. Bayer, V. Sokolova, W. Hansen, T. Sparwasser, U. Dittmer, M. Epple, J. Buer, A. M. Westendorf, *Retrovirology* **2016**, *13*, 24; b) T. Knuschke, O. Rotan, W. Bayer, S. Kollenda, J. Dickow, K. Sutter, W. Hansen, U. Dittmer, K. S. Lang, M. Epple, J. Buer, A. M. Westendorf, *Front. Immunol.* **2018**, *8*, 614.

[62] V. Sokolova, O. Rotan, J. Klesing, P. Nalbant, J. Buer, T. Knuschke, A. M. Westendorf, M. Epple, *J. Nanopart. Res.* **2012**, *14*, 910.

[63] a) B. Neuhaus, A. Frede, A. M. Westendorf, M. Epple, *J. Mater. Chem. B* **2015**, *3*, 7186 – 7193; b) B. Neuhaus, B. Tosun, O. Rotan, A. Frede, A. M. Westendorf, M. Epple, *RSC Adv.* **2016**, *6*, 18102 – 18112; c) S. Chernousova, M. Epple, *Gene Ther.* **2017**, *24*, 282 – 289.

[64] a) M. Keeney, J. J. J. P. van den Beucken, P. M. van der Kraan, J. A. Jansen, A. Pandit, *Biomaterials* **2010**, *31*, 2893 – 2902; b) C. Hadjicharalambous, D. Kozlova, V. Sokolova, M. Epple, M. Chatzinikolaidou, *J. Biomed. Mater. Res. A* **2015**, *103*, 3834 – 3842; c) T. Tenkumo, J. R. Vanegas Sáenz, K. Nakamura, Y. Shimizu, V. Sokolova, M. Epple, Y. Kamano, H. Egusa, T. Sugaya, K. Sasaki, *Mater. Sci. Eng. C* **2018**, *92*, 172 – 183.

[65] a) F. Pittella, M. Zhang, Y. Lee, H. J. Kim, T. Tockary, K. Osada, T. Ishii, K. Miyata, N. Nishiyama, K. Kataoka, *Biomaterials* **2011**, *32*, 3106 – 3114; b) A. Frede, B. Neuhaus, R. Klopfleisch, C. Walker, J. Buer, W. Müller, M. Epple, A. M. Westendorf, *J. Control. Release* **2016**, *222*, 86 – 96; c) T. Tenkumo, L. Rojas-Sánchez, J. R. Vanegas Sáenz, T. Ogawa, M. Miyashita, N. Yoda, O. Prymak, V. Sokolova, K. Sasaki, M. Epple, *Acta Biomater.* **2020**, *105*, 263 – 279.

[66] a) T. Knuschke, W. Bayer, O. Rotan, V. Sokolova, M. Wadwa, C. J. Kirschning, W. Hansen, U. Dittmer, M. Epple, J. Buer, A. M. Westendorf, *Nanomedicine* **2014**, *10*, 1787 – 1798; b) D. Damm, L. Rojas-Sánchez, H. Theobald, V. Sokolova, R. T. Wyatt, K. Überla, M. Epple, V. Temchura, *Nanomaterials* **2019**, *9*, 1389.

[67] L. Rojas-Sánchez, K. Loza, M. Epple, *Materialia* **2020**, *12*, 100773.

[68] J. Conde, J. T. Dias, V. Grazú, M. Moros, P. V. Baptista, J. M. de la Fuente, *Front. Chem.* **2014**, *2*, 48.

[69] C. Zilker, D. Kozlova, V. Sokolova, H. Yan, M. Epple, K. Uberla, V. Temchura, *Nanomedicine* **2017**, *13*, 173 – 182.

[70] M. Kopp, U. W. Aufderhorst, M. Alt, U. Dittmer, A. M. Eis-Hubinger, B. Giebel, M. Roggendorf, M. Epple, A. Krawczyk, *Nanomedicine* **2019**, *16*, 138 – 148.

[71] H. C. Kolb, M. G. Finn, K. B. Sharpless, *Angew. Chem. Int. Ed.* **2001**, *40*, 2004 – 2021; *Angew. Chem.* **2001**, *113*, 2056 – 2075.

[72] V. Sokolova, D. Kozlova, T. Knuschke, J. Buer, A. M. Westendorf, M. Epple, *Acta Biomater.* **2013**, *9*, 7527 – 7535.

[73] S. Kollenda, M. Kopp, J. Wens, J. Koch, N. Schulze, C. Papadopoulos, R. Pöhler, H. Meyer, M. Epple, *Acta Biomater.* **2020**, *111*, 406 – 417.

[74] a) S. Behzadi, V. Serpooshan, W. Tao, M. A. Hamaly, M. Y. Alkawareek, E. C. Dreaden, D. Brown, A. M. Alkilany, O. C. Farokhzad, M. Mahmoudi, *Chem. Soc. Rev.* **2017**, *46*, 4218 – 4244; b) J. Zhao, M. H. Stenzel, *Polym.*

Chem. **2018**, *9*, 259 – 272; c) S. Patel, J. Kim, M. Herrera, A. Mukherjee, A. V. Kabanov, G. Sahay, *Adv. Drug Deliv. Rev.* **2019**, *144*, 90 – 111; d) I. Canton, G. Battaglia, *Chem. Soc. Rev.* **2012**, *41*, 2718 – 2739; e) G. Sahay, D. Y. Alakhova, A. V. Kabanov, *J. Contr. Rel.* **2010**, *145*, 182 – 195; f) N. Oh, J. H. Park, *Int. J. Nanomedicine* **2014**, *9*, 51 – 63.
[75] S. Kumari, S. Mg, S. Mayor, *Cell Res.* **2010**, *20*, 256 – 275.
[76] O. Rotan, K. N. Severin, S. Pöpsel, A. Peetsch, M. Merdanovic, M. Ehrmann, M. Epple, *Beilstein J. Nanotechnol.* **2017**, *8*, 381 – 393.
[77] S. P. Mondéjar, A. Kovtun, M. Epple, *J. Mater. Chem.* **2007**, *17*, 4153 – 4159.
[78] M. A. I. Rasel, S. Singh, T. D. Nguyen, I. O. Afara, Y. Gu, *Sci. Rep.* **2019**, *9*, 5859.
[79] a) F. Scaletti, J. Hardie, Y. W. Lee, D. C. Luther, M. Ray, V. M. Rotello, *Chem. Soc. Rev.* **2018**, *47*, 3421 – 3432; b) V. M. Rotello, *Beilstein J. Org. Chem.* **2016**, *12*, 1638 – 1646; c) M. Ray, R. Tang, Z. Jiang, V. M. Rotello, *Bioconjugate Chem.* **2015**, *26*, 1004 – 1007.
[80] a) R. V. Benjaminsen, M. A. Mattebjerg, J. R. Henriksen, S. M. Moghimi, T. L. Andresen, *Mol. Ther.* **2013**, *21*, 149 – 157; b) G. Creusat, A. S. Rinaldi, E. Weiss, R. Elbaghdadi, J. S. Remy, R. Mulherkar, G. Zuber, *Bioconjugate Chem.* **2010**, *21*, 994 – 1002; c) A. Akinc, M. Thomas, A. M. Klibanov, R. S. Langer, *J. Gene Med.* **2005**, *7*, 657 – 663.
[81] a) K. H. Müller, M. Motskin, A. J. Philpott, A. F. Routh, C. M. Shanahan, M. J. Duer, J. N. Skepper, *Biomaterials* **2014**, *35*, 1074 – 1088; b) M. Motskin, K. H. Möller, C. Genoud, A. G. Monteith, J. N. Skepper, *Biomaterials* **2011**, *32*, 9470 – 9482; c) M. Motskin, D. M. Wright, K. Muller, N. Kyle, T. G. Gard, A. E. Porter, J. N. Skepper, *Biomaterials* **2009**, *30*, 3307 – 3317; d) D. Proudfoot, Y. Dautova, D. Kozlova, M. Epple, M. D. Bootman, *Atherosclerosis* **2016**, *244*, e9 – e10; e) Y. Dautova, D. Kozlova, J. N. Skepper, M. Epple, M. D. Bootman, D. Proudfoot, *PLoS ONE* **2014**, *9*, e97565.
[82] S. Neumann, A. Kovtun, I. D. Dietzel, M. Epple, R. Heumann, *Biomaterials* **2009**, *30*, 6794 – 6802.
[83] a) S. R. Paital, N. B. Dahotre, *Mater. Sci. Eng. R* **2009**, *66*, 1 – 70; b) K. L. Gerlach, D. Niehues, *Mund Kiefer Gesichtschir.* **2007**, *11*, 131 – 137; c) C. K. G. Spies, S. Schnurer, T. Gotterbarm, S. Breusch, *Arch. Orthop. Trauma Surg.* **2009**, *129*, 979 – 988; d) D. Busenlechner, S. Tangl, B. Mair, G. Fugger, R. Gruber, H. Redl, G. Watzek, *Biomaterials* **2008**, *29*, 3195 – 3200; e) M. W. Laschke, K. Witt, T. Pohlemann, M. D. Menger, *J. Biomed Mater. Res. B* **2007**, *82*, 494 – 505.
[84] a) A. K. MacMillan, F. V. Lamberti, J. N. Moulton, B. M. Geilich, T. J. Webster, *Int. J. Nanomed.* **2014**, *9*, 5627 – 5637; b) R. Detsch, D. Hagmeyer, M. Neumann, S. Schaefer, A. Vortkamp, M. Wuelling, G. Ziegler, M. Epple, *Acta Biomater.* **2010**, *6*, 3223 – 3233.
[85] a) A. Peetsch, M. Epple, *Mater. Wiss. Werkstofftechn.* **2011**, *42*, 131 – 135; b) Z. Wang, Y. Sa, S. Sauro, H. Chen, W. Xing, X. Ma, T. Jiang, Y. Wang, *J. Dent.* **2010**, *38*, 400 – 410; c) M. Hannig, C. Hannig, *Nat. Nanotechnol.* **2010**, *5*, 565 – 569; d) J. Enax, M. Epple, *Oral Health Prev. Dent.* **2018**, *16*, 7 – 19.
[86] T. J. Levingstone, S. Herbaj, N. J. Dunne, *Nanomaterials* **2019**, *9*, 1570.
[87] S. M. Moghimi, A. C. Hunter, J. C. Murray, *Pharmacol. Rev.* **2001**, *53*, 283 – 318.
[88] D. Kozlova, M. Epple, *BioNanoMaterials* **2013**, *14*, 161 – 170.
[89] G. Caracciolo, O. C. Farokhzad, M. Mahmoudi, *Trends Biotechnol.* **2017**, *35*, 257 – 264.
[90] a) M. Germain, F. Caputo, S. Metcalfe, G. Tosi, K. Spring, K. K. O. Åslund, A. Pottier, R. Schiffelers, A. Ceccaldi, R. Schmid, *J. Controlled Release* **2020**, *326*, 164 – 171; b) R. van der Meel, E. Sulheim, Y. Shi, F. Kiessling, W. J. M. Mulder, T. Lammers, *Nat. Nanotechnol.* **2019**, *14*, 1007 – 1017.
[91] M. A. Khan, V. M. Wu, S. Ghosh, V. Uskoković, *J. Colloid Interface Sci.* **2016**, *471*, 48 – 58.
[92] a) R. A. Perez, M. P. Ginebra, M. Spector, *J. Mater. Sci. Mater. Med.* **2011**, *22*, 887 – 897; b) Y. Liu, T. Wang, F. He, Q. Liu, D. Zhang, S. Xiang, S. Su, J. Zhang, *Int. J. Nanomed.* **2011**, *6*, 721 – 727; c) B. Mostaghaci, B. Loretz, R. Haberkorn, G. Kickelbick, C. M. Lehr, *Chem. Mater.* **2013**, *25*, 3667 – 3674; d) A. Gigante, M. Li, S. Junghanel, C. Hirschhauser, S. Knauer, C. Schmuck, *MedChemComm* **2019**, *10*, 1692 – 1718; e) H. Liang, X. B. Zhang, Y. F. Lv, L. Gong, R. W. Wang, X. Y. Zhu, R. H. Yang, W. H. Tan, *Acc. Chem. Res.* **2014**, *47*, 1891 – 1901.
[93] F. L. Graham, A. J. van der Eb, *Virology* **1973**, *52*, 456 – 467.
[94] a) S. Bisso, S. Mura, B. Castagner, P. Couvreur, J. C. Leroux, *Eur. J. Pharm. Biopharm.* **2019**, *142*, 142 – 152; b) S. Chernousova, J. Klesing, N. Soklakova, M. Epple, *RSC Adv.* **2013**, *3*, 11155 – 11161; c) C. Schlickewei, T. O. Klatte, Y. Wildermuth, G. Laaff, J. M. Rueger, J. Ruesing, S. Chernousova, W. Lehmann, M. Epple, *J. Mater. Sci. Mater. Med.* **2019**, *30*, 15; d) J. R. Vanegas Sáenz, T. Tenkumo, Y. Kamano, H. Egusa, K. Sasaki, *PLoS One* **2017**, *12*, e0188347.
[95] A. Kovtun, R. Heumann, M. Epple, *Bio-Med. Mater. Eng.* **2009**, *19*, 241 – 247.
[96] a) V. Uskoković, D. P. Uskokovic, *J. Biomed. Mater. Res. B* **2011**, *96*, 152 – 191; b) J. Tang, J. Y. Chen, J. Liu, M. Luo, Y. J. Wang, X. W. Wei, X. Gao, B. L. Wang, Y. B. Liu, T. Yi, A. P. Tong, X. R. Song, Y. M. Xie, Y. Zhao, M. Xiang, Y. Huang, Y. Zheng, *Int. J. Pharm.* **2012**, *431*, 210 – 221; c) Y. Xie, Y. Chen, M. Sun, Q. Ping, *Curr. Pharm. Biotechnol.* **2013**, *14*, 918 – 925.
[97] a) D. Olton, J. Li, M. E. Wilson, T. Rogers, J. Close, L. Huang, N. P. Kumta, C. Sfeir, *Biomaterials* **2007**, *28*, 1267 – 1279; b) C. E. Pedraza, D. C. Bassett, M. D. McKee, V. Nelea, U. Gbureck, J. E. Barralet, *Biomaterials* **2008**, *29*, 3384 – 3392.
[98] S. Heinemann, M. Gelinsky, H. Worch, T. Hanke, *Orthopade* **2011**, *40*, 761 – 773.
[99] R. Khalifehzadeh, H. Arami, *ACS Biomater. Sci. Eng.* **2019**, *5*, 3201 – 3211.
[100] M. D. Krebs, E. Salter, E. Chen, K. A. Sutter, E. Alsberg, *J. Biomed. Mater. Res. A* **2010**, *92*, 1131 – 1138.
[101] a) J. Kurreck, *Angew. Chem. Int. Ed.* **2009**, *48*, 1378 – 1398; *Angew. Chem.* **2009**, *121*, 1404 – 1426; b) P. Gao, X. Zhang, H. Wang, Q. Zhang, H. Li, Y. Li, Y. Duan, *Oncotarget* **2016**, *7*, 2855 – 2866; c) X. C. Xu, Z. H. Li, X. Q. Zhao, L. Keen, X. D. Kong, *Regener. Biomater.* **2016**, *3*, 187 – 195.
[102] M. S. Shim, Y. J. Kwon, *FEBS J.* **2010**, *277*, 4814 – 4827.
[103] a) Y. H. Weng, Q. Q. Huang, C. H. Li, Y. F. Yang, X. X. Wang, J. Yu, Y. Y. Huang, X. J. Liang, *Mol. Ther. Nucl. Acids* **2020**, *19*, 581 – 601; b) B. Kim, J. H. Park, M. J. Sailor, *Adv. Mater.* **2019**, *31*, 1903637; c) W. Ho, X. Q. Zhang, X. Y. Xu, *Adv. Healthcare Mater.* **2016**, *5*, 2715 – 2731.
[104] a) E. V. Giger, B. Castagner, J. Raikkonen, J. Mönkkönen, J.-C. Leroux, *Adv. Healthcare Mater.* **2013**, *2*, 134 – 144; b) M. T. Haynes, L. Huang, *Adv. Genet.* **2014**, *88*, 205 – 229; c) J. Tang, L. Li, C. B. Howard, S. M. Mahler, L. Huang, Z. P. Xu, *J. Mater. Chem. B* **2015**, *3*, 6805 – 6812.
[105] T. Devarasu, R. Saad, A. Ouadi, B. Frisch, E. Robinet, P. Laquerrière, J. C. Voegel, T. Baumert, J. Ogier, F. Meyer, *J. Mater. Chem. B* **2013**, *1*, 4692 – 4700.
[106] F. Pittella, K. Miyata, Y. Maeda, N. Nishiyama, K. Kataoka, *J. Control. Release* **2012**, *161*, 868 – 874.
[107] L. A. Tobin, Y. Xie, M. Tsokos, S. I. Chung, A. A. Merz, M. A. Arnold, G. Li, H. L. Malech, K. F. Kwong, *Biomaterials* **2013**, *34*, 2980 – 2990.
[108] Q. Wang, L. Qin, Y. Sun, Y. Duan, *J. Nanopart. Res.* **2014**, *16*, 1 – 9.
[109] a) W. Zhou, A. Moguche, D. Chiu, K. Murali-Krishna, F. Baneyx, *Nanomedicine* **2014**, *10*, 571 – 578; b) Y. Lin, X. Wang, X. Huang, J. Zhang, N. Xia, Q. Zhao, *Expert. Rev. Vaccines* **2017**, *16*, 895 – 906; c) R. Pati, M. Shevtsov, A. Sonawane, *Front. Immunol.* **2018**, *8*, 2224; d) V. Sokolova, T. Knuschke, A. Kovtun, J. Buer, M. Epple, A. M. Westendorf, *Biomaterials* **2010**, *31*, 5627 – 5633.
[110] T. Knuschke, V. Sokolova, O. Rotan, M. Wadwa, M. Tenbusch, W. Hansen, P. Staeheli, M. Epple, J. Buer, A. M. Westendorf, *J. Immunol.* **2013**, *190*, 6221 – 6229.
[111] P. Sahdev, S. Podaralla, R. S. Kaushik, O. Perumal, *J. Biomed. Nanotechnol.* **2013**, *9*, 132 – 141.
[112] a) R. Khalifehzadeh, H. Arami, *Nanoscale* **2020**, *12*, 9603 – 9615; b) T. Knuschke, M. Epple, A. M. Westendorf, *Hum. Vaccin. Immunother.* **2014**, *10*, 164 – 169; c) D. Kozlova, V. Sokolova, M. Zhong, E. Zhang, J. Yang, W. Li, Y. Yang, J. Buer, A. M. Westendorf, M. Epple, H. Yan, *Virol. Sinica* **2014**, *29*, 1 – 7.
[113] D. Chiu, W. Zhou, S. Kitayaporn, D. T. Schwartz, K. Murali-Krishna, T. J. Kavanagh, F. Baneyx, *Bioconjug. Chem.* **2012**, *23*, 610 – 617.
[114] T. Morcol, P. Nagappan, S. J. D. Bell, A. G. Cawthon, *AAPS PharmSciTech* **2019**, *20*, 315.
[115] a) K. Hasna, S. S. Kumar, M. Komath, M. R. Varma, M. K. Jayaraj, K. R. Kumar, *Phys. Chem. Chem. Phys.* **2013**, *15*, 8106 – 8111; b) L. Degli Esposti, F. Carella, A. Adamiano, A. Tampieri, M. Iafisco, *Drug Dev. Ind. Pharm.* **2018**, *44*, 1223 – 1238; c) M. P. Nikolova, M. S. Chavali, *Bioact. Mater.* **2019**, *4*, 271 – 292; d) Y. Li, C. Liao, S. C. Tjong, *Nanomaterials* **2019**, *9*, 590; e) L. Morejón, J. A. Delgado, A. A. Ribeiro, M. V. de Oliveira, E. Mendizábal, I. García, A. Alfonso, P. Poh, M. van Griensven, E. R. Balmayor, *Int. J. Mol. Sci.* **2019**, *20*, 1790; f) S. Heng, Z. Lu, Q. Liu, T.

Jiang, M. He, F. Song, J. Zhao, L. Zheng, *Mater. Sci. Eng. C* **2020**, *110*, 110691.
[116] a) S. Vieira, S. Vial, R. L. Reis, J. M. Oliveira, *Biotechnol. Prog.* **2017**, *33*, 590–611; b) D. N. Heo, W. K. Ko, H. R. Lee, S. J. Lee, D. Lee, S. H. Um, J. H. Lee, Y. H. Woo, L. G. Zhang, D. W. Lee, I. K. Kwon, *J. Colloid Interface Sci.* **2016**, *469*, 129–137.
[117] a) T. Kumai, N. Yui, K. Yatabe, C. Sasaki, R. Fujii, M. Takenaga, H. Fujiya, H. Niki, K. Yudoh, *Int. J. Nanomed.* **2019**, *Volume 14*, 1283–1298; b) E. J. Sheehy, D. J. Kelly, F. J. O'Brien, *Mater. Today Bio.* **2019**, *3*, 100009; c) K. T. Shalumon, C. Y. Kuo, C. B. Wong, Y. M. Chien, H. A. Chen, J. P. Chen, *Polymers (Basel)* **2018**, *10*, 620.
[118] a) R. Logith Kumar, A. Keshav Narayan, S. Dhivya, A. Chawla, S. Saravanan, N. Selvamurugan, *Carbohydr. Polym.* **2016**, *151*, 172–188; b) A. Rogina, L. Pribolsan, A. Hanzek, L. Gomez-Estrada, G. G. Ferrer, I. Marijanovic, M. Ivankovic, H. Ivankovic, *Polymer* **2016**, *98*, 172–181; c) V. Sokolova, K. Kostka, K. T. Shalumon, O. Prymak, J. P. Chen, M. Epple, *J. Mater. Sci. Mater. Med.* **2020**, *31*, 102.
[119] a) M. I. Hassan, T. Sun, N. Sultana, *J. Nanomater.* **2014**, *2014*, 209049; b) S. K. L. Levengood, M. Q. Zhang, *J. Biomed. Mater. Res. B Appl. Biomater.* **2014**, *2*, 3161–3184; c) T. Muthukumar, A. Aravinthan, J. Sharmila, N. S. Kim, J. H. Kim, *Carbohydr. Polym.* **2016**, *152*, 566–574; d) Z. L. Wang, Y. Wang, Y. Ito, P. B. Zhang, X. S. Chen, *Sci. Rep.* **2016**, *6*, 20770.
[120] D. Huang, B. He, P. Mi, *Biomater. Sci.* **2019**, *7*, 3942–3960.
[121] a) I. L. Aanei, A. M. ElSohly, M. E. Farkas, C. Netirojjanakul, M. Regan, S. T. Murphy, J. P. O'Neil, Y. Seo, M. B. Francis, *Mol. Pharm.* **2016**, *13*, 3764–3772; b) Q. F. Liu, M. Zhou, P. L. Li, G. Ku, G. Huang, C. Li, S. L. Song, *Contrast Media Mol. Imaging* **2016**, *11*, 475–481; c) E. Voulgari, A. Bakandritsos, S. Galtsidis, V. Zoumpourlis, B. P. Burke, G. S. Clemente, C. Cawthorne, S. J. Archibald, J. Tucek, R. Zboril, V. Kantarelou, A. G. Karydas, K. Avgoustakis, *J. Control. Release* **2016**, *243*, 342–356; d) X. L. Gao, L. H. Guo, J. Q. Li, H. E. Thu, Z. Hussain, *J. Control. Release* **2018**, *292*, 29–57.
[122] M. Jauregui-Osoro, P. A. Williamson, A. Glaria, K. Sunassee, P. Charoenphun, M. A. Green, G. E. D. Mullen, P. J. Blower, *Dalton Trans.* **2011**, *40*, 6226–6237.
[123] V. Lobaz, R. Konefał, J. Pánek, M. Vlk, J. Kozempel, M. Petřík, Z. Novy, S. Gurská, P. Znojek, P. Štěpánek, M. Hrubý, *Colloids Surf. B Biointerfaces* **2019**, *179*, 143–152.
[124] A. L. C. Maia, C. H. Cavalcante, M. G. F. de Souza, C. d. A. Ferreira, D. Rubello, S. Chondrogiannis, V. N. Cardoso, G. A. Ramaldes, A. L. B. de Barros, D. C. F. Soares, *Nucl. Med. Commun.* **2016**, *37*, 775–782.
[125] a) T. Lammers, F. Kiessling, M. Ashford, W. Hennink, D. Crommelin, G. Storm, *Nat. Rev. Mater.* **2016**, *1*, 16069; b) S. K. Golombek, J. N. May, B. Theek, L. Appold, N. Drude, F. Kiessling, T. Lammers, *Adv. Drug Delivery Rev.* **2018**, *130*, 17–38.

Manuscript received: December 9, 2020
Accepted manuscript online: February 12, 2021
Version of record online: March 16, 2021

