Hybrid Materials

Fluorescent Inorganic-Organic Hybrid Nanoparticles


Dedicated to Professor Bernt Krebs on the occasion of his 80th birthday
1. Introduction

Medicine and molecular biology belong to the most fascinating and most challenging areas of nanoparticle application. Here, nanoparticles generally address two different subjects: Diagnosis and therapy. Aiming at diagnosis, nanoparticles can serve as contrast agents for all kinds of imaging techniques including optical imaging (OI), photoacoustic imaging (PAI), magnetic resonance imaging (MRI), ultrasonic imaging (US), computed tomography (CT), scintigraphy (SC), or positron emission tomography (PET). In regard of therapy, nanoparticles are promising carriers for drug delivery and release. For instance, this includes analgesic, anti-tumor, anti-inflammatory particles as powerful tags for fluorescence detection and optical imaging. IOH-NPs are suitable for multimodal imaging, and the visualization of various types of tissue on different scales of resolution. However, OI essentially requires suitable fluorescent contrast agents. Even more interesting than single-modality contrast agents is the option of multimodal detection to combine the specific assets of different imaging techniques for the visualization of various types of tissue on different scales of resolution, for the translation from preclinical to clinical imaging, or the translation of preoperative to intraoperative imaging.

This review is specifically focused on fluorescent nanoparticles as powerful tags for fluorescence detection and OI. In general, various requirements are prerequisite to contrast agents for any application in basic medical research and life sciences, including: i) Low toxicity and sufficient biocompatibility; ii) Easy detectability with standard hardware equipment; iii) Highly specific signals to prevent optical overlap with autofluorescence from cells and tissue; iv) Deep tissue penetration of the irradiation used for excitation and emission; v) Straightforward synthesis of contrast agents with low material complexity. In this regard, three types of fluorescent nanoparticles have been yet applied most often: i) Semiconductor-type quantum dots (Q-dots); ii) Up-converting nanoparticles; iii) Immobilized organic dyes. Aiming at fluorescent nanoparticles, Q-dots beyond doubt represent the most prominent and most widely applied class of inorganic nanoparticles. They are designated by unrivalled brightness, intense size-depending emission, likewise in the UV to IR spectral regime, superior photostability, and low photo-
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mean particle diameter $<$ 10 nm) and the color purity of emission (i.e. maximum deviation from the mean diameter $\pm$ 0.5 nm).\textsuperscript{[16]} Moreover, high crystallinity and high purity are necessary to exclude defect-driven loss processes. The latter requirement also demands core-shell structures (e.g. CdSe@ZnS) with a luminescent core (e.g. CdSe) that is coated by a non-luminescent shell (e.g. ZnS) to eliminate the solid-to-liquid surface of the luminescent core.\textsuperscript{[16]} The above requirements – size control, low defect level, core-shell structure – lead to high efforts for chemical synthesis. The applicability of Q-dots, finally, suffers from additional inherent drawbacks such as harmful elements (e.g. Cd\textsuperscript{2+}), sensitivity to hydrolysis, and the hydrophobic properties of the as-prepared material (e.g. due to alkyl-terminalized surfaces).\textsuperscript{[17]} All in all, advanced strategies of synthesis and surface conditioning are needed to obtain state-of-the-art water-dispersible core-shell Q-dots showing intense emission.\textsuperscript{[18]} As an alternative class of fluorescent nanoparticles, moreover, carbon dots (C-dots) came up recently and turned out as very promising due to their improved biocompatibility.\textsuperscript{[19]}

Similar demands regarding crystallinity, purity, and surface conditioning with core-shell structures also apply to up-converting nanoparticles (e.g. NaYF\textsubscript{4}:Er,Yb@NaYF\textsubscript{4}) to avoid all kinds of defects, which otherwise reduce the emission intensity.\textsuperscript{[20]} Up-conversion – meaning the absorption of two or more photons of lower energy (e.g. IR) followed by the emission of one photon at higher energy (e.g. green) – is established by photon-cascade processes on the precisely defined energy levels of rare-earth-metal ions (e.g., Er\textsuperscript{3+}, Yb\textsuperscript{3+}).\textsuperscript{[21]} Up-conversion-based fluorescence guarantees low background and excellent tissue penetration. On the other hand, up-conversion materials suffer from poor absorption and high photon density. The latter is needed for sufficient excitation (e.g. by monochromatic laser light) of the quantum-mechanically forbidden f-f transitions on rare-earth-metal ions.

Besides inorganic nanoparticles, such as Q-dots and up-converting materials, fluorescent organic dyes in aqueous solution are, of course, most often used as tags for fluorescence detection and optical imaging.\textsuperscript{[10]} Thus, a great number of fluorescent dyes showing emission from the blue to the infrared spectral regime has been discovered since the beginning of fluorescence microscopy in the early 20\textsuperscript{th} century to analyze all kinds of biological probes.\textsuperscript{[22]} However, fluorescent organic dyes often do not meet the demands on high emission intensity, sufficient photostability, and chemical resistance under the conditions of investigation.\textsuperscript{[22a,22b]} In particular, low intensity and rapid photobleaching are severe limitations of many fluorescent organic dyes. To evade these intrinsic weaknesses, fluorescent organic dyes – most often including derivatives of rhodamine, cyanine, squaraine, boron-dipyromethene, porphyrin, phthalocyanine, etc.\textsuperscript{[23]} – were attached to or encapsulated in inorganic matrices (e.g., silica, calcium phosphate),\textsuperscript{[24]} metalorganic frameworks and coordination polymers,\textsuperscript{[25]} organic polymers (e.g., polyglycolic acid/PGA, polyactic acid/PLA, poly(lactic-co-glycolic) acid/PLGA, polycaprolactone/PLC, chitosan),\textsuperscript{[26a,26b]} as well as liposomes and dendrimers.\textsuperscript{[27]} However, surface-attached dyes hold the risk of abrasive debonding. Encapsulation of fluorescent organic dyes in a matrix, on the other hand, is typically performed via micro-emulsion techniques, which limits the amount of available material.\textsuperscript{[28]} Moreover, the concentration of the fluorescent dye in relation to the inert matrix is usually low ($\leq$ 5 wt-%). The limited number of fluorescent centers per nanoparticle volume, however, reduces the emission intensity and increases the threat of photobleaching.

Although fascinating chemical compositions and material structures of fluorescent nanoparticles were realized, aspects such as the emission intensity, photostability, biocompatibility, or biodegradability still need further improvement. The complexity and great number of constituents of certain nanoparticulate architectures, in fact, can be a limitation in itself since synthesis and material are becoming more and more complex and expensive. Aiming at medical application, furthermore, all individual constituents as well as all their combinations might need clinical approval.\textsuperscript{[29]} Based on the above discussed state-of-the-art, we present inorganic-organic hybrid nanoparticles (IOH-NPs) as a novel concept and class of fluorescent nanoparticles for biomedical issues. We have explored these IOH-NPs since 2008,\textsuperscript{[30]} with the intention to identify uncomplicated, low-cost nanoparticles showing intense emission and high biocompatibility.

2. Fluorescent Inorganic-Organic Hybrid Nanoparticles (IOH-NPs)

The concept of the IOH-NPs is illustrated with phosphate-based IOH-NPs and sulfonate-based IOH-NPs as examples. This includes the chemical synthesis, the material composition as well as the luminescence properties with the specific focus on biomedical application.

2.1. Material Concept

Aiming at uncomplex, low-cost nanoparticles for biomedical use, we intended to perform the synthesis in water only, since the addressed area of application – for obvious reasons – is limited to water. Consequently, the aspired nanoparticles need to be insoluble in water. In terms of biocompatibility, phosphates seemed reasonable as they are essential for the energy metabolism of almost all cells.\textsuperscript{[31]} In this regard, the most insoluble metal phosphate in water is zirconium phosphate, which is well-known in qualitative analysis to prove the presence either of zirconium or phosphate.\textsuperscript{[32]} The precipitation is highly indicative since zirconium phosphate is the only insoluble metal phosphate even in hydrochloric acid at low pH (i.e. pH $\leq$ 2).

Although the qualitative test reaction is described in many textbooks, the chemical composition of the aqueous precipitate at room temperature is still not clear. Typically, the composition is denoted as Zr\textsubscript{2}(PO\textsubscript{4})\textsubscript{5} or Zr(HPO\textsubscript{4})\textsubscript{2}$\times$H\textsubscript{2}O.\textsuperscript{[33,34]} With aqueous conditions at room temperature and at moderate pH (4 to 9), in fact, neither Zr\textsuperscript{4+} nor PO\textsubscript{4}\textsuperscript{3-} are likely as dissolved species due
to their high acidity or alkalinity, respectively. The expected species rather are \( [\text{ZrO}]^{2+} \) and \( [\text{HPO}_4]^{2-} \) or \( [\text{H}_2\text{PO}_4]^- \). Aiming at nanoparticles, a composition \( [\text{ZrO}]^{2+} [\text{HPO}_4]^{2-} \) is indeed formed upon injection of an aqueous solution of \( \text{ZrOCl}_2 \) into an aqueous solution of \( \text{Na}_2(\text{HPO}_4) \) (Figure 1a). It needs to be noted that \( [\text{ZrO}]^{2+} [\text{HPO}_4]^{2-} \) – similar to all IOH-NPs presented in the following – is amorphous and does not show any Bragg peak. This complicates the determination of composition and structure, hence requiring different independent tools to prove the adopted composition (e.g. \( \text{Zr}:\text{P} \) ratio of \( [\text{ZrO}]^{2+} [\text{HPO}_4]^{2-} \) determined to 1:1; see 2.4 for details).

Although not showing any luminescence, \( [\text{ZrO}]^{2+} [\text{HPO}_4]^{2-} \) is the initial point of all IOH-NPs and the origin of a platform of compounds with different compositions.\[34]\ To obtain fluorescent IOH-NPs, the simple hydrogen phosphate anion is replaced by a phosphorous acid with a fluorescent organic dye (\( R_{\text{org}} \)) bound via a \( \text{P} \cdot \text{O} \cdot \text{C} \) ester bond, resulting in a final composition \( [\text{ZrO}]^{2+} [R_{\text{org}}\text{OPO}_3]^2- \) (Figure 1b).\[34,35] With this composition, it is obvious that \( R_{\text{org}} \) in principle, can comprise a great number of different fluorescent dyes. Essentially, the fluorescent organic dye needs to contain a phosphate functionality to obtain insoluble nanoparticles in water.\[34,35]\  

Phosphate-based IOH-NPs \( [\text{ZrO}]^{2+} [R_{\text{org}}\text{OPO}_3]^2- \) are composed of \( [\text{ZrO}]^{2+} \) as an inorganic cation and \( [R_{\text{org}}\text{OPO}_3]^2- \) as a fluorescent organic anion.\[36]\ Due to charge neutrality, cation and anion are available in 1:1 ratio (in the case of identical charges) and in molar quantities. Such saline composition is simple to similar sodium chloride that consists of equimolar amounts of \( \text{Na}^+ \) cations and \( \text{Cl}^- \) anions. The saline composition also accounts for a specific advantage of the IOH-NPs, which is an unprecedentedly high dye load (70–85 wt-%). Due to the intermixing of the inorganic part (the cation) and the organic part (the anion) on the molecular level, the nanoparticles are designated as “inorganic-organic hybrid nanoparticles”, according to the Latin origin: “hybrid”; crossbred.\[34,35]\  

The predominant role of \( [\text{ZrO}]^{2+} \) in the IOH-NPs is to guarantee their insolubility in water, which is prerequisite for obtaining nanoparticles as well as for the intended water-limited biomedical application.\[34,35]\ A comparable approach was yet only reported for bisphosphonates, which serve as organic bridging ligands in coordination polymers.\[35b,35c]\  

Zirconium as a metal cation is known for its low toxicity and has been clinically approved in the US and the EU, for instance, in the form of sodium zirconium cyclosilicate (Lokelma, AstraZeneca) for the treatment of hyperkalemia\[^{[38]}\] or in the case of different zirconium complexes as antiperspirants.\[^{[39]}\] The fluorescent organic anion \( [R_{\text{org}}\text{OPO}_3]^2- \) entails the fluorescence features of the IOH-NPs.\[^{[34,35]}\] In regard of the above considerations, \( [\text{ZrO}]^{2+} [R_{\text{org}}\text{OPO}_3]^2- \) IOH-NPs already offer the following advantages: i) Uncomplex aqueous synthesis; ii) Low material complexity; iii) High dye load leading to intense emission; iv) Wide variability of fluorescent organic anions.

### 2.2. Phosphate-based IOH-NPs

Phosphate-based IOH-NPs with a general chemical composition \( [\text{ZrO}]^{2+} [R_{\text{org}}\text{OPO}_3]^2- \) are here introduced with the exemplary system \( [\text{ZrO}]^{2+} ([\text{HPO}_4])^{x-} ([\text{FMN}]^{2-})^{1-x} \) (\( x = 0–1 \)) containing the inorganic cation \( [\text{ZrO}]^{2+} \) as well as the anions \( [\text{HPO}_4]^{2-} \) and \( [\text{FMN}]^{2-} \).\[^{[34]}\] FMN represents the fluorescent organic anion flavin mononucleotide and is a derivative of vitamin \( \text{B}_2 \) (Figure 2a).\[^{[40]}\]

![Figure 1](/image.png)

**Figure 1.** Scheme illustrating the synthesis of: **a)** \( [\text{ZrO}]^{2+} [\text{HPO}_4]^{2-} \) NPs and **b)** fluorescent inorganic-organic hybrid nanoparticles (IOH-NPs) with a general composition \( [\text{ZrO}]^{2+} [R_{\text{org}}\text{OPO}_3]^2- \).

![Figure 2](/image.png)

**Figure 2.** \( [\text{ZrO}]^{2+} ([\text{FMN}]^{2-}) \) IOH-NPs (FMN: flavin mononucleotide) with: **a)** Scheme of synthesis; **b)** Particle size according to SEM; **c)** Aqueous suspensions at daylight and with blue-light excitation; **d)** Emission and excitation spectra; **e)** Optical microscopy; **f)** Fluorescence microscopy imaging in cells, and **g)** in mice after intradermal injection of nanoparticles (Cy5-NHS intravascular vessel stain), (modified reproduction from ref. [34]).
vigorously stirring at slightly elevated temperatures (55°C). Moreover, the dye anion was added with 5–10 mol-% excess to guarantee anion-terminated particle surfaces. Subsequent to the synthesis, the IOH-NPs were washed by repeated redispersion and centrifugation in/from water and/or ethanol to remove remaining salts and starting materials. After the purification, the [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} IOH-NPs can be easily suspended in polar solvents (e.g., water, ethanol, diethylene glycol) or biological buffers (e.g., HEPES, aqueous dextran). The as-prepared suspensions are colloidal stable over several months and can contain up to 10 wt-% of the IOH-NPs.[34]

Suspensions of [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} IOH-NPs are transparent and – depending on their concentration – exhibit a yellow to orange color (Figure 2c). Upon blue-light excitation (LED with \(\lambda_{\text{max}}=465\) nm), bright green emission (480–650 nm) is observed with its maximum at 530 nm (Figure 2d).[34] According to dynamic light scattering (DLS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM), the as-prepared [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} IOH-NPs have a mean hydrodynamic diameter of 39(±12) nm and a primary particle diameter of 25–40 nm, respectively (Figure 2b).[34] The size distribution indicates that all particles are below 100 nm. In this regard it needs to be taken into account that the synthesis was performed in water without any specific surface agent for controlling nucleation and growth. The determination of the chemical composition of the IOH-NPs is generally challenging, and hence, described in detail in a separate chapter (see 2.4).

The fluorescence of [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} as expected – originates from the FMN anion. Quantum size effects are naturally not involved. Thus, excitation and emission of the [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} IOH-NPs (in suspension) can be directly compared to free FMN in solution (Figure 2d).[34] The quantum yield of FMN even in solution is comparably low (about 30%).[35] Interestingly, the quantum yield of [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-} with 28±2% is identical when considering the experimental significance. Although the [FMN]^{2-} anions – and thus, the fluorescence centers – are in close proximity, no concentration quenching was observed. This finding can be ascribed to the amorphous nature of the IOH-NPs that do not exhibit any periodic packing and long-ranging order. Actually, this random distribution of [FMN]^{2-} guarantees intense emission. In particular for [ZrO]^{2+}([FMN]^{2-}, the enormous dye load of 81 wt-% and the resulting quasi-infinite reservoir of fluorescent centers per volume of each single nanoparticle leads to intense spotlight-type emission.[34] Even certain photobleaching does not noticeably reduce the emission intensity since a great number of fluorescence centers still remains intact (see 2.3: Figure 6c).

Since the IOH-NP concept – including aqueous synthesis, uncomplex material composition and structure, high load of FMN per nanoparticle (81 wt-%) – aims at biomedical application, [ZrO]^{2+}[\{HPO_4\}_{2-x}(FMN)_x]^{2-}, and especially [ZrO]^{2+}([FMN]^{2-}, were evaluated in vitro and in vivo studies regarding fluorescence and imaging performance. Indeed, [ZrO]^{2+}([FMN]^{2-} IOH-NPs show massive uptake into cells. They exhibit high biocompatibility and show intense green emission (Figure 2f).[34] In cells or mice the green fluorescence is typically stable over several hours and disappears after 2–3 days with complete dissolution of the IOH-NPs. Toxic or allergic effects – even after a period of two months – were not observed.[34]

According to TEM and electron-energy loss spectroscopy (EELS), the [ZrO]^{2+}([FMN]^{2-} IOH-NPs localize exclusively in vesicles (Figure 2e). They do not colocalize with mitochondria or nuclei. Thus, [ZrO]^{2+}([FMN]^{2-} IOH-NPs appear to be a suitable tool for staining viable structures.

In spite of the discussed advantages of the [ZrO]^{2+}([HPO_4]_{2-x}(FMN)_x]^{2-} IOH-NPs, FMN-related green emission is less favorable for biomedical application. On the one hand, cells and tissue show green autofluorescence themselves so that a considerable background is present in addition to the green emission of the IOH-NPs. Moreover, green emission and – even more important – blue light, required for excitation, exhibit low tissue penetration.[36,44] Biomedical issues and optical imaging-based applications in animal models and in humans, therefore, require long-wavelength emission rather in the far-red and near-infrared range, since the light absorption by water and hemoglobin is minimal in this spectral range, resulting in optimal tissue penetration. Taking the composition [ZrO]^{2+}([FMN]^{2-}, [PUP]_{2-}, and the concept of the IOH-NPs as a general strategy, phosphate-functionalized fluorescent organic dyes with other luminescence properties as FMN are needed.

Indeed, the IOH-NP concept allows using further fluorescent organic anions and creating a platform of materials. Here, we show the expansion of the concept to [ZrO]^{2+}([PUP]_{2-}, [ZrO]^{2+}([MFP]_{2-}, [ZrO]^{2+}([RRP]_{2-}, and [ZrO]^{2+}([DUT]_{2-} with PUP: phenyl-umbelliferon phosphate, MFP: methylfluorescein phosphate, RRP: resorufin phosphate, and DUT: DY-647 uridine triphosphate (Figure 3a).[35,36] All these additional phosphate-based IOH-NPs are insoluble in water, too, and can be prepared via aqueous synthesis. They exhibit mean particle diameters of 20–40 nm at high colloidal stability (Figure 4a). [ZrO]^{2+}([PUP]_{2-}, [ZrO]^{2+}([MFP]_{2-}, [ZrO]^{2+}([RRP]_{2-}, and [ZrO]^{2+}([DUT]_{2-} show full color emission in the blue (380–600 nm, \(\lambda_{\text{max}}=458\) nm), green (460–700 nm, \(\lambda_{\text{max}}=518\) nm), red (550–700 nm, \(\lambda_{\text{max}}=584\) nm) and near-infrared (630–780 nm, \(\lambda_{\text{max}}=675\) nm) spectral regime (Figure 3a).
Due to the quasi-infinite number of fluorescence centers (dye content up to 85 wt-%), all these phosphate-based IOH-NPs again show intense spotlight emission in aqueous suspensions.

To illustrate the biomedical performance, [ZrO]2-[MFP]2-, [ZrO]2-[RRP]2- and [ZrO]2-[DUT]2- were incubated in vitro with murine alveolar macrophages (MHS) cell lines (50 μg IOH-NPs per mL of cell culture medium) to analyze uptake and fluorescence. After incubation (5 h at 37 °C), the internalization of the IOH-NPs is clearly demonstrated and becomes even more evident after 24 h (Figure 4b). The granularly structured fluorescence in the macrophages indicates the presence of the IOH-NPs, which was also confirmed by EELS, showing a similar granular structure for the localization of zirconium in the macrophages. Controls with macrophages incubated with the IOH-NPs at 4 °C at reduced metabolic activity as well as macrophages cultivated without IOH-NPs do not show any comparable fluorescence. Furthermore, no relevant toxic effects of [ZrO]2-[MFP]2-, [ZrO]2-[RRP]2- and [ZrO]2-[DUT]2- were observed with concentrations up to 250 μM. After subcutaneous injection in nude mice, the emission is also clearly visible in vivo (Figure 4c). Thus, the capability of phosphate-based IOH-NPs for in vivo optical imaging is confirmed as a proof-of-the-concept.

2.3. Sulfonate-based IOH-NPs

Although phosphate-based IOH-NPs [ZrO]2-[RnoserO]3+ already stand for a broad platform of materials with different fluorescent organic anions [RnoserO]3+, the number of commercially available fluorescent organic dyes containing a phosphate functionality is limited. Moreover, phosphate-functionalyzed dyes, and in particular those showing red and infrared emission, are often extremely expensive (up to 500 € per 1 mg). In contrast, almost all conventional fluorescent organic dyes are commercially available with sulfonate functions. Functionalization of aromatic organic molecules by sulfonate groups is synthetically straightforward and often used to make such dyes soluble in water. As a consequence, almost all organic dye systems – such as coumarins, rhodamines, oxazines, cyanines, etc. – are available with one or more sulfonate group. Introducing such sulfonate-based fluorescent anions [Rnoser(SO3)]n- into the IOH-NP concept could, of course, dramatically broaden the platform of fluorescent nanomaterials.

In contrast to phosphate-based fluorescent anions [RnoserO]3-, however, sulfonate-based fluorescent anions [Rnoser(SO3)]n- do not form any insoluble compound upon addition of [ZrO]2+ in water. Taking binary metal sulfates as most suitable reference systems, BaSO4 and PbSO4 are actually known as the most insoluble sulfates in water. Both cations Ba2+ and Pb2+, however, are also known as severely harmful to animate beings. Anyway, both cations are not suitable though they also do not form insoluble compounds with sulfonate-based fluorescent anions [Rnoser(SO3)]n-. This can be rationalized based on the monovalent charge of the sulfonate group in comparison to the divalent sulfate ion, which reduces the Coulomb interaction significantly. Since the charge of a sulfonate group is fixed, the only option is to choose a cation having a comparable radius as Ba2+/Pb2+ (149/133 pm) but a higher charge. In this regard, La3+ (117 pm) is promising and indeed results in the formation of insoluble compounds together with sulfonate-based fluorescent anions [Rnoser(SO3)]n-.[46] Due to the similarity of the lanthanides, in principle, this holds for all rare-earth-metal ions Ln3+ (Ln3+: La3+ to Lu3+). From all these ions, La3+ is interesting due to its low cost. Aiming at novel contrast agents, however, Gd3+ is even more interesting as it opens the option of multimodal imaging via the fluorescence of the dye anion and the paramagnetism of Gd3+. [6]

Sulfonate-based IOH-NPs with a general composition M3+n/2 [Rnoser(SO3)]n-, [M(OH)]2n/2 [Rnoser(SO3)]n-, or [MO]n/2 [Rnoser(SO3)]n- with M: rare-earth metal, and most preferentially with La3+ or Gd3+, can indeed contain a great number of different sulfonate-based dye anions.[6,48] As an illustrative example, we take M3+[AMA]3-(M = La, Gd) with amaranth red (AMA) as the sulfonate-based fluorescent dye anion (Figure 5a).[6] which is also known as E123, C.I. 16185, Acid Red 27, C-Red 46, Echtrot D, or Food
Red 9.\textsuperscript{[49]} AMA is widely used, for instance, in food industry, and can be considered as a less harmful fluorescent dye in comparison to many azo-dyes.

The synthesis of M$^3{\text{[AMA]}}^3^-$ (M = La, Gd) is comparable to the synthesis of the phosphate-based IOH-NPs and performed by injecting an aqueous solution of LaCl$_3\times 7$H$_2$O or GdCl$_3\times 6$H$_2$O to an aqueous solution of Na$_3$(AMA). Again, general aspects of colloid chemistry according to LaMer’s model\textsuperscript{[50]} need to be considered to control particle nucleation and particle growth and to obtain nanoparticles and colloidaly stable suspensions (Figure 5c). Hence, injection was again performed whilst vigorously stirring at slightly elevated temperature (55°C). Moreover, the dye anion was used with 10 mol-% excess in relation to the cation to guarantee anion-terminated particle surfaces. After synthesis and purification, M$^3{\text{[AMA]}}^3^-$ IOH-NPs can be easily suspended in solvents, such as water, ethanol, diethylene glycol, or biological buffers like HEPES or aqueous dextran. According to the chemical composition, La$^3^+$ [AMA]$^3^-$ and Gd$^3^+$ [AMA]$^3^-$ contain extraordinarily high dye loads of 79 wt-% and 77 wt-% [AMA]$^3^-$, respectively.\textsuperscript{[48]}

The particle diameter of the as-prepared M$^3{\text{[AMA]}}^3^-$ IOH-NPs was at first measured in DEG, which is known for excellent stabilization of nanoparticles via surface coordination.\textsuperscript{[53]} Here, a mean hydrodynamic diameter of 68 ± 10 nm with narrow size distribution was observed (Figure 5c). In water, nanoparticles generally show larger hydrodynamic radii due to a rigid layer of adsorbed solvent molecules. Based on the high polarity and extensive hydrogen bonding networks, this rigid solvent layer is largely expanded in water.\textsuperscript{[51]} Thus, a mean hydrodynamic diameter of 105 ± 30 nm was obtained (Figure 5c). Finally, overview SEM images show uniform spherical particles with a mean diameter of 47 ± 10 nm, which was calculated by statistical evaluation of 130 nanoparticles (Figure 5b). Zeta potential analysis of La$^3^+$ [AMA]$^3^-$ and Gd$^3^+$ [AMA]$^3^-$ show negative charging at −12.5 mV in the biologically most relevant pH range of pH 4 to 8 (Figure 5d).\textsuperscript{[48]} The resulting electrostatic stabilization is beneficial for both controlling the particle size as well as suppressing agglomeration.

Suspensions of La$^3^+$ [AMA]$^3^-$ and Gd$^3^+$ [AMA]$^3^-$ exhibit a brilliant red color (Figure 6a) and an intense red emission upon excitation by green light (glass fiber with green filter, $\lambda_{\text{exc}} = 555$ nm) (Figure 6a).\textsuperscript{[48]} Fluorescence spectroscopy indicates strong absorption at 450 to 650 nm and emission at 650 to 800 nm peaking at $\lambda_{\text{em}} = 700$ nm (Figure 6b). Such luminescence features are ideal for biomedic application in terms of low background from autofluorescence and deep penetration of tissue.\textsuperscript{[44]}

M$^3^+$ [AMA]$^3^-$ IOH-NPs show higher photostability than dissolved AMA (Na$_3$(AMA)) in solution at identical concentration (33 mmol/mL AMA). UV-irradiation of M$^3^+$ [AMA]$^3^-$ suspensions and of dissolved AMA ($\lambda_{\text{exc}} = 310$ nm, 15 h) displays continuous photobleaching with only 27% of pristine emission intensity remaining for dissolved AMA (Figure 6c). In contrast, M$^3^+$ [AMA]$^3^-$ suspensions show almost constant emission intensity over the complete period of irradiation.\textsuperscript{[48]} The photostability of the IOH-NPs is even more remarkable when compared to conventional nanoparticle systems with fluorescent organic dyes encapsulated in inorganic or polymeric matrices (Table 1). They all show considerable photobleaching even on short timescales. The high photostability of the M$^3^+$ [AMA]$^3^-$ IOH-NPs can be attributed, on the one hand, to the extremely high dye load so that certain photobleaching at the particle surface leaves the emission intensity more-or-less unaffected. Despite of strong absorption in the visible (450–700 nm), on the other hand, the IOH-NPs exhibit a high reflectivity in the UV spectral regime (< 450 nm).\textsuperscript{[48]} In particular, the absorption of high-energy light is lower, resulting in a reduced formation of reactive oxygen species (ROS), and therefore, a higher photostability. SiO$_2$ or Ca$_3$(PO$_4$)$_2$ as widely applied inorganic matrices to encapsulate fluorescent organic dyes, are much less UV-reflective, resulting in a significant photobleaching at daylight (Table 1).

Besides the AMA-based fluorescence, Gd$^{3+}$-related paramagnetism of seven unpaired electrons is expected for Gd$^{3+}$ [AMA]$^{3-}$. Indeed, powder samples of the IOH-NPs can be already attracted by a bar magnet (Figure 7a). Magnetic measurements quantify the magnetic properties of the as-prepared Gd$^{3+}$ [AMA]$^{3-}$ to an effective magnetic moment of 6.83(1) μ$_{B}$ per Gd atom and a Weiss constant of $θ _W = 4.3(5)$ K (Figure 7b).\textsuperscript{[48]} These data are comparable to Gd$^{3+}$-based MRI contrast agents, such as the coordination complexes Gd-DPTA (DPTA: diethylenetriaminepentaacetate) and Gd-DOTA (DOTA: 1,4,7,10-tetraazacyclododecane-N,N’,N’’,N’’’-tetraacetic acid),\textsuperscript{[55]} which are clinically applied with about 0.1 mmol Gd$^{3+}$ per kg of body weight.\textsuperscript{[56]} An equal Gd$^{3+}$ content would require about 70 mg of Gd$^{3+}$ [AMA]$^{3-}$ per kg of body weight. In comparison to standard Gd-DPTA and Gd-DOTA, it must be noted that Gd$^{3+}$...
Photobleaching of conventional nanocomposites with fluorescent organic dyes encapsulated in inorganic or polymer matrices.

Table 1.

<table>
<thead>
<tr>
<th>Encapsulated fluorescent dye</th>
<th>Matrix material</th>
<th>Time of UV irradiation</th>
<th>Intensity after irradiation</th>
<th>Half-lifetime of emission</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy3</td>
<td>Ca3(PO4)2</td>
<td>300 s</td>
<td>97%</td>
<td>Not measured</td>
<td>[24f] [52]</td>
</tr>
<tr>
<td>Cy3</td>
<td>SiO2</td>
<td>7 h</td>
<td>98%</td>
<td>Not measured</td>
<td>[24g]</td>
</tr>
<tr>
<td>Cy5</td>
<td>SiO2</td>
<td>200 s</td>
<td>25%</td>
<td>60 s</td>
<td>[53]</td>
</tr>
<tr>
<td>Tetramethylrhodamine isothio-cyanate/TRITC</td>
<td>SiO2</td>
<td>30 min</td>
<td>88%</td>
<td>Not measured</td>
<td>[24d]</td>
</tr>
<tr>
<td>Fluorescein iso-thiocyanate/FITC</td>
<td>SiO2</td>
<td>30 min</td>
<td>70%</td>
<td>Not measured</td>
<td>[24d]</td>
</tr>
<tr>
<td>Indocyanine green/ICG</td>
<td>Ca3(PO4)2</td>
<td>633 s</td>
<td>50%</td>
<td>633 s</td>
<td>[24h]</td>
</tr>
<tr>
<td>Indocyanine green/ICG</td>
<td>PLGA[48]</td>
<td>60 min</td>
<td>52%</td>
<td>60 min</td>
<td>[26b] [54]</td>
</tr>
</tbody>
</table>

M+[AMA]+, PVK[a], (M: La, Gd) +[FMN]2+[AMA]3+, Acid Red 97 ([AR97] 2[C0]n/2, [Gd(OH)]2[C0]n/3, [M(OH)]2[C0]n/2, or Evans Blue ([EB] 4[C0]n/3, Nuclear Fast Red ([NFR] 2[C0]n/3, or Iodine Blue ([IB] 4[C0]n/3, containing the sulfonate-based fluorescent anions Chicago Sky Blue ([CSB] 4[C0]n/3), Direct Blue 71 ([DB71] 4[C0]n/3), Nuclear Fast Red ([NFR] 2[C0]n/3, Acid Red 97 ([AR97] 2[C0]n/3, or Evans Blue ([EB] 4[C0]n/3. In particular, this holds for Chicago Sky Blue,[35]

As discussed for phosphate-based IOH-NPs ([ZrO] 2[C0]3n/2, [Gd(OH)]2[C0]n/3, M+[AMA]3+ (M = La, Gd) can be also considered as first representative of a wider platform of sulfonate-based IOH-NPs with a general composition ([M]3n+[M(OH)]2[C0]n/3, [M(OH)]2[C0]n/3, [Gd(OH)]2[C0]n/3, and [Gd(OH)]3[C0]n/3) containing the sulfonate-based fluorescent anions Chicago Sky Blue ([CSB] 4[C0]n/3), Direct Blue 71 ([DB71] 4[C0]n/3), Nuclear Fast Red ([NFR] 2[C0]n/3, Acid Red 97 ([AR97] 2[C0]n/3, or Evans Blue ([EB] 4[C0]n/3. These fluorescent dyes are commercially available and used in solution for staining in cell biology and histology.[9,10] In particular, this holds for Chicago Sky Blue,[35]

[AMA]3+ - IOH-NPs also show fluorescence so that they become suitable for multimodal imaging (OI, MRI).

To further evaluate the applicability of Gd3+[AMA]3+ as a contrast agent, the IOH-NPs were incubated with MHS macrophages.[36] Similar to [ZrO]2+[FMN]3+, a massive uptake of the IOH-NPs was demonstrated by fluorescence microscopy after 5 h of incubation at 37°C (Figure 8a), whereas internalization is strongly reduced at 4°C. The viability of the macrophages is unaffected by the uptake of the Gd3+[AMA]3+ IOH-NPs (Figure 8b). Only at high concentrations (>200 μg/mL), the cell viability is decreasing, which, in fact, can be related to a reduced concentration of culture medium. This finding points to a good biocompatibility of the Gd3+[AMA]3+ IOH-NPs.[44]
Nuclear Fast Red, \[^{[58]}\] and Evans Blue. \[^{[61]}\] Similar to \(\text{M}^3\), \[^{[63]}\] the sulfonate-based IOH-NPs exhibit intense absorption leading to the blue color of \([\text{Gd(OH)}_2]^2\) and \([\text{Gd(OH)}_2]^3\), as well as to the orange to red color of \([\text{Gd(OH)}_2]^2\) and \([\text{Gd(OH)}_2]^3\) (Figures 9d). \[^{[58]}\] Most interestingly, fluorescence was yet only based on >100 nanoparticles. \[^{[a]}\] Statistical average based on >100 nanoparticles. \[^{[b]}\] See 3.1; \[^{[c]}\] See 3.2.

### 2.4. Chemical Composition

Proving the chemical composition of the IOH-NPs is challenging. First of all, it needs to be noticed that all IOH-NPs are non-crystalline. They do not show any specific Bragg peak in X-ray diffraction or electron diffraction experiments. \[^{[34,35,48,58]}\] Consequently, crystal structures of the compounds are unknown. On the other hand, the absence of periodically ordered arrays is advantageous, if not essential in regard of the fluorescence of the IOH-NPs. In the case of crystalline structures with periodically aligned fluorescent organic anions, severe concentration quenching would have been expected that could eradicade the emission of the IOH-NPs partly or completely. \[^{[13]}\]

In order to elucidate the chemical composition of the IOH-NPs, different analytical methods need to be involved to gain insights at different levels of priority. Obviously, it is the highest priority to prove the presence of the fluorescent organic anion \([R_{\text{NFR}},\text{OP}_{\text{O}},\text{O}]^2\) or \([R_{\text{N}},\text{S(O)}_2]_{\text{S}}^3\). Besides fluorescence spectroscopy, here, Fourier-transformed infrared (FT-IR) spectroscopy is indicative, as exemplarily shown for \(\text{Gd}^{3+}[\text{AMA}]^{3−}\) (Figure 10a). \[^{[46]}\] A comparison with the starting material \(\text{Na}_3\text{AMA}\) as reference shows all characteristic vibrations of AMA, including \(\nu(\text{O}−\text{H}): 3600−3000\ \text{cm}^{-1}, \nu(\text{N}−\text{N}): 1370\ \text{cm}^{-1}, \nu(\text{C}−\text{N}−\text{C}): 1100−1500\ \text{cm}^{-1}\), which are found as expected in the FT-IR spectrum of \(\text{Na}_3\text{AMA}\). In contrast, the IOH-NPs do not contain these functional groups.

#### Table 3. Excitation and emission of sulfonate-based IOH-NPs.

<table>
<thead>
<tr>
<th>IOH-NP composition</th>
<th>Excitation range (nm)</th>
<th>Emission range (nm)</th>
<th>Emission (\lambda_{\text{max}}) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{Gd}^{3+}[\text{AMA}]^{3−})</td>
<td>400−650</td>
<td>650−800</td>
<td>700</td>
</tr>
<tr>
<td>([\text{Gd(OH)}_2]^2) ([\text{CSB}]^4)</td>
<td>240−460</td>
<td>400−550</td>
<td>437</td>
</tr>
<tr>
<td>([\text{Gd(OH)}_2]^3) ([\text{DB71}]^4)</td>
<td>320−440</td>
<td>400−550</td>
<td>444</td>
</tr>
<tr>
<td>([\text{Gd(OH)}_2]^2) ([\text{NFR}]^4)</td>
<td>400−580</td>
<td>520−740</td>
<td>748</td>
</tr>
<tr>
<td>([\text{Gd(OH)}_2]^3) ([\text{AR97}]^4)</td>
<td>550−730</td>
<td>550−640</td>
<td>592</td>
</tr>
<tr>
<td>([\text{Gd(OH)}_2]^2) ([\text{EB}]^4)</td>
<td>350−640</td>
<td>700−880</td>
<td>762</td>
</tr>
<tr>
<td>([\text{Gd}^3]) ([\text{ICG}]_{\text{C6}}^4)</td>
<td>700−820</td>
<td>780−840</td>
<td>810</td>
</tr>
<tr>
<td>([\text{La}_3]^+) ([\text{TPPS}_4]^3−)</td>
<td>380−600</td>
<td>540−700</td>
<td>585</td>
</tr>
<tr>
<td>([\text{Gd}^{3+}[\text{APCS}]_{\text{C6}}^4) [^{[a]}]</td>
<td>250−400, 550−720</td>
<td>650−770</td>
<td>686</td>
</tr>
</tbody>
</table>

\[^{[a]}\] See 3.1; \[^{[b]}\] See 3.2.
Accordingly, the thermal combustion reaction can be qualitatively proven by energy dispersive X-ray spectroscopy (EDXS). Quantification is usually not possible via EDXS since the presence of the inorganic metal cation is important and the charges of cation and anion already suggest a cation-to-anion ratio of 1:1, which is validated by performing thermogravimetry (TG) to obtain the total organics content. Prior to TG, the as-prepared Gd$^{3+}$[AMA]$^{3-}$ IOH-NPs were dried in vacuo at room temperature for 8 h to remove all adsorbed volatiles (e.g. water). Thereafter, TG shows a total weight loss of 72% up to a temperature of 1000 °C, which corresponds well to the calculated weight loss of 69% of total organics combustion for the assumed composition Gd$^{3+}$[AMA]$^{3-}$ (Figure 10b). Moreover, the thermal remnant of TG analysis was identified via XRD as Gd$_2$O$_2$(SO$_4$). Accordingly, the thermal combustion reaction can be rationalized as follows:[46]

$$2\text{Gd}^{3+}[\text{C}_{28}\text{H}_{33}\text{N}_2\text{O}_{11}\text{S}_3]^{3+} + 53/2\text{O}_2 \rightarrow$$
$$\text{Gd}_2\text{O}_2(\text{SO}_4) + 4\text{CO}_2 + 11\text{H}_2\text{O} + 2\text{N}_2 + 5\text{SO}_2$$

In addition to TG analysis, the composition of Gd$^{3+}$[AMA]$^{3-}$ can be independently verified by elemental analysis (EA) resulting in C/H/N/S contents of: 32 wt-% C, 3 wt-% H, 4 wt-% N, and 12 wt-% S. Within the experimental error, these data are well in accordance with the calculated values: 36 wt-% C, 2 wt-% H, 4 wt-% N, and 14 wt-% S. Taking all analytical data together (FT-IR, EDX, TG, EA), the chemical composition of Gd$^{3+}$[AMA]$^{3-}$ is reliably substantiated.[48] With similar investigations the chemical composition of other phosphate- or sulfonate-based IOH-NPs was determined, as well (Tables 4, 5).

For different reasons the determination of the chemical composition of the IOH-NPs still remains challenging. Thus, the thermal decomposition is complicated in the case of the phosphate-based IOH-NPs due to the encapsulation of the organic content within the metal phosphate formed during thermal decomposition. Actually, such effect is well-known for flame retarding materials. They often contain phosphates to encapsulate organic materials, and thereby, to increase the ignition temperature.[51] For analysis of the IOH-NPs, slow heating needs to be performed to guarantee the total combustion of all organic constituents via TG and EA. For all IOH-NPs, and in particular for sulfonate-based IOH-NPs, oxide atoms and/or hydroxide and water molecules could be coordinated to the metal cation. This is another option to vary the chemical composition of the IOH-NPs as expressed by the formula [M]$^{1-}[\text{RDye}(\text{SO}_3)n]n$. Due to its low weight (relative to the inorganic cation and the fluorescent dye anion), the coordination of charged species like O$^-$ or OH$^-$ to the metal cation cannot be reliably evidenced based on the above described analytical techniques. On the other hand, the presence of O$^-$ or OH$^-$ would naturally change the net charge of the inorganic cation, and thereby also the ratio of inorganic cation and fluorescent dye anion. This molar ratio, however, can be precisely determined based on TG and EA. Finally, non-charged H$_2$O molecules could be coordinated to the inorganic cation. As they do not influence the net charge of the cation or the charge neutrality of the IOH-NPs, and as they only have low weight, such H$_2$O coordination (most probably of 1–2 H$_2$O molecules per formula unit) cannot be excluded.

Although non-crystalline, finally, the IOH-NPs can be assumed to exhibit layer-type structures. Especially in the case of zirconium phosphates, such as Zr(HPO$_4$)$_2$×H$_2$O, layered arrangements have been described.[64] Layered structures were also often observed for zirconium in combination with organophosphates, and especially, organophosphonates.[65] Actually, these compounds are highly relevant as flame retardants.[66]

### Table 4. Chemical composition of phosphonate-based IOH-NPs according to EA (C,H,N,S content) and TG (total organic combustion).

<table>
<thead>
<tr>
<th>Compound</th>
<th>C content (%) (EA)</th>
<th>H content (%) (EA)</th>
<th>N content (%) (EA)</th>
<th>Zr:P ratio (EDX)</th>
<th>Weight loss (%) (TG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[ZrO$^2+$(RRP)$^2-$]</td>
<td>26 (34)</td>
<td>3 (2)</td>
<td>3 (4)</td>
<td>1.2:1</td>
<td>43 (48)</td>
</tr>
<tr>
<td>[ZrO$^2+$(FMN)$^2-$]</td>
<td>34 (38)</td>
<td>/</td>
<td>9 (10)</td>
<td>1:1</td>
<td>61 (64)</td>
</tr>
<tr>
<td>[ZrO$^2+$(MFP)$^2-$]</td>
<td>42 (47)</td>
<td>4 (3)</td>
<td>0 (0)</td>
<td>1:1</td>
<td>62 (64)</td>
</tr>
<tr>
<td>[ZrO$^2+$(PUP)$^2-$]</td>
<td>32 (42)</td>
<td>3 (2)</td>
<td>0 (0)</td>
<td>1:1</td>
<td>50 (54)</td>
</tr>
</tbody>
</table>

[a] Note that the composition of [ZrO$^2+$(DUT)]$^2-$ was not studied in detail due to the very high cost of [DUT]$^-$ (about 500 € for 1 mg).
Nevertheless, it must be stated that the structural characterization of zirconium phosphates is still lacking, in general.

3. Specific Properties of Inorganic-Organic Hybrid Nanoparticles (IOH-NPs)

Subsequent to the illustration of the material concept of the IOH-NPs and after having shown their feasibility for fluorescence detection and optical imaging, we now address more specific functionalities of selected IOH-NPs. This includes [GdO\(^{3+}\)][ICG\(^{-}\)] for multimodal imaging,\(^{[69]}\) Gd\(_{3}\)([AlPcS\(_{4}\)]\(^{n+}\)) (AlPcS\(_{4}\): aluminium(III) chloride phthalo-cyanine tetrasulfonate) showing singlet-oxygen generation,\(^{[70]}\) as well as the dissolution of the IOH-NPs and their use for drug delivery and drug release.

3.1. Multimodal Imaging

To combine the specific assets of different imaging techniques (e.g., resolution, imaging of different types of tissue) and/or to translate preoperative to intraoperative imaging (and vice versa) suitable contrast agents for multimodal imaging are highly interesting.\(^{[13]}\) In this regard, materials with different functionalities were often integrated with high virtuosity into complex nanoarchitectures. This includes, for instance, superparamagnetic iron oxide nanoparticles (SPIONs) and inorganic fluorescent nanoparticles (e.g., Q-dots, lanthanide-doped oxides) or molecular fluorescent dyes (e.g., coumarins, rhodamines, oxazines, cyanines) that were encapsulated in or attached to inorganic or organic matrices (e.g., silica, calcium phosphate, polymers, liposomes, dendrimers).\(^{[3,10]}\) As discussed before, the complexity of the resulting contrast agents and the sheer number of constituents can be a restriction in itself as all constituents and combinations must be verified individually for clinical approval. In practice, in vivo application becomes the more prohibitive that the greater the complexity and the more multi-component the employed materials.\(^{[29]}\) Moreover, the amount of active contrast agent can be very low in an inert matrix as majority component, which reduces the detection limit.\(^{[24–27]}\)

In regard to the IOH-NPs, we already discussed optical absorption and fluorescence (see 2.2, 2.3), and we also pointed to Gd\(^{3+}\) as an optional cation in sulfonate-based IOH-NPs (see 2.3) to implement the IOH-NPs in regard of OI and MRI. Besides imaging, optical absorption, fluorescence, and magnetism can be also used to locate the IOH-NPs in cells and tissue and to determine their in vivo and in vitro behavior and dissolution. A most interesting type of IOH-NP for multimodal imaging, in this regard, is [GdO\(^{3+}\)][ICG\(^{-}\)], which consists of equimolar amounts of paramagnetic gadolinium as the inorganic cation and [ICG\(^{-}\)] as the organic fluorescent dye anion (Figure 11a).\(^{[69]}\)

Again, an aqueous synthesis was applied to prepare [GdO\(^{3+}\)][ICG\(^{-}\)] using GdCl\(_{3}\)·6H\(_{2}\)O and Na[ICG] as the starting materials. [GdO\(^{3+}\)][ICG\(^{-}\)] contains 81 wt-% [ICG\(^{-}\)] and can be easily suspended in polar solvents (e.g., water, ethanol, diethylene glycol) or biological media (e.g., HEPES, aqueous dextran) with concentrations up to 10 mg/mL. The analytical characterization regarding particle size and chemical composition was performed as described before (see 2.3/2.4: Tables 2, 5). Accordingly, SEM and DLS show mean particle diameters of 49 ± 8 nm and 50 ± 9 nm, respectively, with narrow size distribution (Figure 11b) and a zeta potential of −20 to −35 mV at pH 6–8 (Figure 11c).\(^{[69]}\)

Aiming at OI, ICG is optimal for biomedical application in many aspects. On the one hand, its strong visible absorption (700–820 nm) and its NIR emission (780–840 nm) are ideal for deep-tissue penetration minimizing the absorbance by water and hemoglobin (Figure 11d).\(^{[16,44]}\) Moreover, ICG is well-tolerated (LD\(_{50}\): 50–80 mg/kg), approved for clinical use, and already widely used in the clinic for histology.\(^{[24–27]}\) Furthermore, ICG is cheap (about 50 € per 1 g)\(^{[72]}\) in comparison to many alternative commercial red- and infrared-emitting dyes that are conventionally used for OI.\(^{[21,45]}\) On the other hand, ICG as a dissolved molecule has several weaknesses such as: i) Rapid binding to human serum albumin and high-density lipoproteins causing agglomeration and rapid clearance via the liver; ii) Very short circulation time (half-life of only 2–4 min in mice); iii) Low fluorescence quantum yield (only about 5% in water); iv) Low chemical stability under physiological conditions due to fast biodegradation; v) Rapid photobleaching under light exposure.\(^{[71,73]}\) Similar to other fluorescent organic dyes, ICG was also often encapsulated in stabilizing matrices (e.g., organic polymers, liposomes, micelles, silica) to overcome these limitations.\(^{[71,44]}\) Moreover, ICG has been intercalated in layered double hydroxides (LDHs).\(^{[71]}\) Again, such inert matrices reduce the available amount of ICG per nanoparticle and lower the intrinsically weak emission intensity even further.

In contrast to ICG solutions, suspensions of [GdO\(^{3+}\)][ICG\(^{-}\)] IOH-NPs show intense emission due to the high ICG load (81 wt-%) and the great number of fluorescent centers per nanoparticle (see 2.3: Table 3). Naturally, [GdO\(^{3+}\)][ICG\(^{-}\)] (in suspension) exhibits identical fluorescence features as ICG (in solution).\(^{[109]}\) Accordingly, the dark green aqueous suspensions show deep red emission upon visible light excitation (Fig-
[GdO]^{3+} MHS macrophages at 37°C. 

In vitro studies with MHS macrophages show an excellent uptake of [GdO]^{3+} [ICG] as indicated by their intense emission (Figure 12a). Massive uptake of IOH-NPs was again observed upon incubation at 37°C, whereas only minimal uptake was observed at 4°C. This observation indicates an active acquisition of [GdO]^{3+} [ICG] by the macrophages. It is to be noticed that the IOH-NPs were coated by dextran to improve the membrane permeability and cell uptake. 

Despite massive internalization of [GdO]^{3+} [ICG] IOH-NPs, the metabolic activity and viability of the MHS cells – according to cell proliferation assays – remain almost unaffected (Figure 12b). When comparing [GdO]^{3+} [ICG] (in suspension) with ICG (in solution) or Gd-DOTA/Gd-DTPA (in solution) as references, the viability of MHS cells treated with the IOH-NPs turned out as only slightly lower as compared to dissolved ICG and significantly higher than for the standard MRI contrast agents Gd-DOTA/Gd-DTPA (Figure 12b). Naturally, this comparison was performed at similar concentration of ICG and/or Gd. In regard of its performance as a contrast agent, finally, it must be noted that only [GdO]^{3+} [ICG] is multimodal and suitable as contrast agent for OI and MRI.

Whereas free ICG (in solution) is typically not suitable for fluorescence detection due to its low emission, it is one of the most promising absorptive contrast agents for PAI. 

Therefore, an evaluation of [GdO]^{3+} [ICG] IOH-NPs in regard of its feasibility for PAI seemed obvious. The performance of [GdO]^{3+} [ICG] (in suspension) indeed turned out as similar to ICG (in solution) at identical ICG concentration (Figure 12c). Blood vessel phantoms, chicken-breast phantoms, and dead-mouse phantoms showed promising signal intensity and depth of detection, indicating the feasibility of [GdO]^{3+} [ICG] IOH-NPs also for PAI. 

Besides in vitro studies, the multimodal features of [GdO]^{3+} [ICG] in terms of MRI and OI were evaluated in vivo. In response to increasing concentrations, the IOH-NPs clearly result in a reduction of the $T_1$-relaxation (Figure 13a). 

The specific relaxivity ($r_1$) per gadolinium at 7 Tesla was determined to 8.0 ± 0.4 mM⁻¹s⁻¹ for dextran-coated [GdO]^{3+} [ICG] . In fact, the relaxivity is even higher than for Gd-DTPA or Gd-DOTA (3–5 mM⁻¹s⁻¹). [6] In vivo, mice were imaged before and 5 h after intravenous injection of [GdO]^{3+} [ICG] IOH-NPs. Images show $T_1$-relaxation time heat maps with a noticeably reduced relaxation in the gall bladder and liver (by 35%). (modified reproduction from ref. [6]).

In parallel with MRI, mice were also imaged in vivo with fluorescence molecular tomography (FMT) and post mortem – after exposing the organs – using fluorescence reflectance imaging (FRI) (Figure 14). Again, [GdO]^{3+} [ICG] IOH-NPs can be clearly detected. Detection via FMT is even possible over a time range of several hours. In combination, FMT and FRI demonstrate that [GdO]^{3+} [ICG] IOH-NPs are suitable as multimodal contrast agents in vitro as well as in vivo for OI, PAI and MRI. Straightforward synthesis and low material complexity of [GdO]^{3+} [ICG] IOH-NPs are additional assets in comparison to many contrast agents discussed in the literature. In the clinics, [GdO]^{3+} [ICG] IOH-NPs could allow to combine the presentation of different types of tissue, for instance, soft tissue via MRI, blood vessels via PAI, and single cells via OI. 

The chemists who conducted the study are acknowledged for their contribution to this work. 

Figure 12. In vitro studies with [GdO]^{3+} [ICG] IOH-NPs: a) Fluorescence images of [GdO]^{3+} [ICG] (10 µg/mL of medium) after 24 h incubation with MHS macrophages at 37°C; b) Metabolic activity of MHS macrophages after 0, 24, 48 and 72 h cultivation with [GdO]^{3+} [ICG] suspensions (dextran-coated); ICG, Gd-DOTA and Gd-DTPA solutions as references (0-200 nM/mL of [GdO]^{3+} [ICG], Gd-DOTA, Gd-DTPA); c) PAI with [GdO]^{3+} [ICG] (suspension) and ICG (solution) at identical concentrations in a dead-mouse phantom (modified reproduction from ref. [69]).

Figure 13. [GdO]^{3+} [ICG] IOH-NPs (dextran-coated) as MR contrast agent: a) Maps of $T_1$-relaxation time calculated for varying concentrations of dextran-coated IOH-NPs; b) Mice imaged before and 5 h after injection with [GdO]^{3+} [ICG] IOH-NPs. Images show $T_1$-relaxation time heat maps with a noticeably reduced relaxation in the gall bladder and liver (by 35%). (modified reproduction from ref. [69]).
The resulting IOH-NPs have a modified reproduction from ref. [70]. The quantum yield of singlet-oxygen (1O2) generation of Gd4+[AlPCS4]3− and La4+[TPPS4]3− suspensions (Figure 15c). Gd4+[AlPCS4]3− and La4+[TPPS4]3− exhibit mean diameters of 47 and 56 nm, respectively (Figure 15b; see 2.3: Table 2). [72,76] Their chemical composition was validated by FT-IR, TG, EDXS and EA (see 2:4: Table 5). Both Gd4+[AlPCS4]3− and La4+[TPPS4]3− also show visible emission, which, in principle, is also sufficient for fluorescence detection and OI (see 2:3: Table 3).

In fact, phthalocyanines as well as porphyrins are well-known for efficient photoactivated 1O2 generation. Both are already discussed for applications such as the selective oxidation in organic synthesis (e.g., cycloadditions, Diels-Alder reactions, Ene reactions, heteroatom oxidations) [79] the degradation of organic molecules and cells (e.g. for water purification) [80] and photodynamic therapy (PDT) (most often used for tumor therapy) [81]. Certain porphyrins and phthalocyanines are already approved in the clinics [81c,d,82]. Aiming at PDT, molecular photosensitizers in solution, however, have certain disadvantages, such as low solubility in water and rapid aggregation under physiological conditions. Both reduce the efficiency of 1O2 production and result in limited membrane permeability and poor cell uptake [83]. Again, it was suggested to immobilize the molecular porphyrins and phthalocyanines on/in nanosized/nanoporous substrates, such as silica and gold nanoparticles as well as carbon nanotubes [81b,83,84] which again leads to only low amounts of the active molecular photocatalyst (typically < 10 wt-%). Moreover, metalorganic frameworks containing porphyrin linkers were presented [85]. For medical application, in particular, encapsulation in vesicles and liposomes or functionalization with hydrophilic capping ligands was established [86]. However, these measures also enhance the material complexity, and any encapsulation/capping, as a matter of fact, blocks the active sites of the photocatalysts.

The feasibility of Gd4+[AlPCS4]3− and La4+[TPPS4]3− for 1O2 production was validated by the DPBF method (DPBF: 1,3-diphenylisobenzofuran) [87] and the iodide method [88]. DPBF is oxidized in the presence of 1O2 as indicated by the vanishing absorption at 420 nm can be easily monitored by UV-Vis spectroscopy and results in a quantum yield of 33% for 1O2 production of Gd4+[AlPCS4]3− and La4+[TPPS4]3− (in suspension). This value matches very well with molecular H4AlPCS4 (35%, in solution) [89]. This finding is even more interesting, since H4AlPCS4 is known for rapid photodegradation in solution. In contrast, Gd4+[AlPCS4]3− IOH-NPs show good photostability without any considerable concentration quenching [70]. Both can be explained, on the one hand, by the great phthalocyanine/porphyrin reservoir, and, on the other hand, by the non-crystallinity of the IOH-NPs that avoids any ordered alignment of the phthalocyanine anions. The quantum yield of 1O2 generation of La4+[TPPS4]3− cannot be performed via the more common DPBF method since the absorption of [TPPS4]3− overlays the DPBF absorption band. Instead, the iodide method...
was applied here,[88] resulting in a quantum yield of 49% for La$_4^+$[TPPS$_4$]$^{3-}$/C$_0$ and La$_4^+$[TPPS$_4$]$^{3-}$/C$_0$ (in suspension), which is again close to the value of molecular H$_2$TPPS$_3$ (51%, in solution).[89]

To determine the photocatalytic performance of Gd$_4^+$[AlPCS$_4$]$^{3-}$ and La$_4^+$[TPPS$_4$]$^{3-}$ (Figure 16a), both were first conceptually evaluated in regard of the photocatalytic degradation of Erichrome Black T (EBT). EBT was chosen as a model dye since its absorption ($\lambda_{max} = 525$ nm) does not overlap that of Gd$_4^+$[AlPCS$_4$]$^{3-}$ ($\lambda_{max} = 670$ nm) and La$_4^+$[TPPS$_4$]$^{3-}$ ($\lambda_{max} = 425$ nm). The photocatalytic degradation was studied with simulated daylight (halogen lamp) as well as with red-light illumination (glass filter with $\lambda > 610$ nm) by comparing Gd$_4^+$[AlPCS$_4$]$^{3-}$ and La$_4^+$[TPPS$_4$]$^{3-}$ (in suspension) with H$_2$AlPCS$_4$ and H$_2$TPPS$_3$ (in solution) as references.[70] Despite of identical concentrations of the photoactive phthalocyanine/porphyrin, the IOH-NPs (in suspension) show significantly higher photodegradation efficiency than the non-charged IOH-NPs.[91] Moreover, the local absorption intensity of the IOH-NPs (in suspension) is higher due to the great number of absorbing centers per volume of each nanoparticle compared to the widely separated H$_2$AlPCS$_4$ and H$_2$TPPS$_3$ molecules (in solution).

With the above described features and performance – including the extremely high phthalocyanine/porphyrin load and the promising photocatalytic effect with daylight illumination – the Gd$_4^+$[AlPCS$_4$]$^{3-}$/C$_0$ and La$_4^+$[TPPS$_4$]$^{3-}$/C$_0$ IOH-NPs can be highly interesting not only for photocatalytic dye degradation but also for PDT.[78] PDT is generally considered as a useful addition to the armory against cancer since it is minimally invasive and non-damaging to healthy tissue. Specifically, PDT is known for treatment of near-surface tumors (e.g., skin, esophagus or intestinal cancer)[90] and intensely discussed for post-surgery treatment to kill cancer cells that may remain after extraction of the solid primary tumor.[91] In view of deep-tissue penetration, the long-wavelength excitation of Gd$_4^+$[AlPCS$_4$]$^{3-}$ (550–720 nm) seems most interesting to PDT and was therefore studied in vitro and in vivo. Gd$_4^+$[AlPCS$_4$]$^{3-}$/H$_2$AlPCS$_4$ (in solution) is already approved for PDT.[92] Based on the paramagnetism of Gd$_4^+$ and the red deep emission of [AlPCS$_4$]$^{3-}$, furthermore, Gd$_4^+$[AlPCS$_4$]$^{3-}$/IOH-NPs are also suitable for multimodal imaging including MRI and OL. With these features, Gd$_4^+$[AlPCS$_4$]$^{3-}$/IOH-NPs can be an advantageous addition to existing nanoparticulate photosensitizers for PDT. So far, this includes inorganic nanoparticles such as TiO$_2$ or ZnO as well as rare-earth based up-converters,[93] which suffer from UV-activation (i.e., TiO$_2$. ZnO) being harmful to cells[94,95] or narrow-line laser-type excitation (i.e. up-conversion via f-f transitions on rare-earth metals).[96,97]

The feasibility of Gd$_4^+$[AlPCS$_4$]$^{3-}$/IOH-NPs was demonstrated in vitro with human liver carcinoma (HePG2) cells and human cervix carcinoma (HeLa) cells.[78] The incubation of cells with dextran-coated Gd$_4^+$[AlPCS$_4$]$^{3-}$/IOH-NPs proves good cellular uptake as indicated by an intense red fluorescence (Figure 17a,b). Moreover, efficient photoactivated $^{1}$O$_2$ generation was evidenced with DCFDA-treated (DCFDA: profluorescent 2',7'-dichlorofluorescein (DCF) produced in free IOH-NPs (20 µM) in HePG2 cells after 24 h of incubation: a) Prior to illumination in daylight and b) after 10 min of illumination with red fluorescence indicating the IOH-NP uptake (nuclei stained with Hoechst 33342); c) Cells treated with DCFDA prior and d) after illumination with green fluorescence of DCF indicating ROS generation. Illumination performed by scanning slides 4-times for 13 min using scan cycles at 670 nm (Pearl Imager, LI-COR Biosciences; N: nucleus; scale bar: 20 µm), (modified reproduction from ref. [78]).

Finally, the phototoxic effect of the Gd₄⁺[AlPCS₄]⁺ IOH-NPs was evaluated in an in vivo zebrafish tumor model. Zebrafishes are generally known as a suitable platform for testing and refining therapies in the preclinical phase of drug development. They are easier to handle than mice and allow a direct readout by OI due to their small size and transparency. In our study, Gd₄⁺[AlPCS₄]⁺ IOH-NPs were first incubated with HeLa-GFP cells expressing green fluorescent proteins (GFP). Thereafter, the IOH-NP-pretreated HeLa-GFP cells were injected into the cardinal vein of zebrafish larvae to induce tumors (Figure 19a). The Gd₄⁺[AlPCS₄]⁺ IOH-NP-pretreated HeLa-GFP cells could be easily detected in the zebrafish larvae by their red and green emission (Figure 19b). Upon light exposure over a certain period of time (10 min, 670 nm), the green emission of the HeLa-GFP cells is significantly decreased, which indicates their reduced viability. Finally, only the red emission of the Gd₄⁺[AlPCS₄]⁺ IOH-NPs remains at the position of cellular debris and necrotic tumors (Figure 19b). HeLa-GFP cells without Gd₄⁺[AlPCS₄]⁺ IOH-NPs were injected into zebrafish larvae as a control and did not show any effect and vanishing of the green emission at all (Figure 19a). As a result, the phototoxic performance of the Gd₄⁺[AlPCS₄]⁺ IOH-NPs is clearly shown in vivo. Specific optimization of parameters – such as the concentration of the IOH-NPs, the duration of illumination, certain repeat treatments – is of course needed to reliably explore the optimal treatment efficiency and to establish therapy protocols.

3.3. Dissolution of IOH-NPs

In cell and mouse studies of all IOH-NPs, and in particular of the phosphate-based [ZrO]⁺³[Rₙ₋₁PO₄]₂⁻ IOH-NPs, the fluorescence was observed to vanish on a timescale of several hours to some days. Typically, no fluorescence and no nanoparticle remains could be identified after 3–4 days. This finding points to a slow dissolution of the IOH-NPs, which can be rationalized upon hydrolysis of the P–O–C phosphorus acid ester bond, resulting in the dissolved species [ZrO]⁺³, [HPO₄]²⁻ and Rₙ₋₁OH (Figure 20). Such dissolution can be triggered by acid or base catalysis as well as by ubiquitous phosphatases in cells and tissue. In fact, such dissolution is ideal in terms of biocompatibility and biodegradability, especially if there is no specific toxicity of the dissolved species and if all species are completely released from cells, tissue and body after certain period of time.

\[ \text{Phosphatase} \]
\[ [ZrO]^{3+} [R_{n-1}OPO_4]^{2-} + H_2O \rightarrow [ZrO]^{2+} + R_{n-1}OH + [HOPO_4]^{2-} \]

Figure 20. Model reaction for the slow metabolic dissolution of [ZrO]⁺³[Rₙ₋₁PO₄]₂⁻ IOH-NPs (modified reproduction from ref. [35]).

Figure 18. In vitro phototoxicity of Gd₄⁺[AlPCS₄]⁺ IOH-NPs as indicated by MTT assays: a) Cells treated with Gd₄⁺[AlPCS₄]⁺ IOH-NPs (green bars) and with dissolved H₃AlPCS₄ (black bars) after 72 h of incubation in darkness; b) Cells treated similarly and with illumination at 670 nm for 2 × 5 min (statistical error bars calculated from n = 6; significance determined according to student’s t-test with p < 0.05; N: nucleus), (reproduction from ref. [78]).

Figure 19. In vivo phototoxicity and imaging of Gd₄⁺[AlPCS₄]⁺ IOH-NP-pretreated HeLa cells in zebrafish larvae after NIR illumination. Larvae were xenografted with GFP expressing and Gd₄⁺[AlPCS₄]⁺ IOH-NPs (5 μM) pretreated HeLa cells. After 24 h, the larvae were illuminated for 10 min at 670 nm and thereafter imaged using fluorescent confocal microscopy: a) GFP expression exited at 488 nm (Argon laser) and the emission detected at 498–540 nm; b) Gd₄⁺[AlPCS₄]⁺ IOH-NPs excited at 635 nm and the emission detected at 644–786 nm. Upon illumination the nuclei were losing GFP expression and the tumors were reduced in size (N: nucleus; scale bar: 200 μm), (modified reproduction from ref. [78]).
Various *in vitro* and *in vivo* studies in the meantime have proven that no specific toxicity or allergic reaction is caused by the IOH-NPs.\cite{24,31,48,58,78} In particular, this holds for the cations \([\text{ZrO}]^{2+}\) and \(\text{Gd}^{3+}/\text{Gd(OH)}^{3+}/\text{GdO}^{2+}\). Even for the latter, a lower toxicity as compared to the standard MRI contrast agents Gd-DOTA and Gd-DTPA was observed.\cite{19,21} A detection of the dissolved species after their release from the IOH-NPs, however, is not straightforward. On the one hand, immediate dilution of the dissolved species in the volume of cells, tissue and body leads to only low concentrations remaining, and thereby hampers the detection. Analyzing \([\text{HPO}_4]^{2-}\) and \(\text{RDye}_\text{OH}\) is difficult anyway due to the ubiquitous physiological availability of phosphate and the rapid metabolic decomposition of the fluorescent dye anion. Here, radio-labeling could be a useful option. Tracking of \([\text{ZrO}]^{2+}\) is easier due to its absence in animate beings. *In vivo* studies indeed show that the pristine amount of injected zirconium in \([\text{ZrO}]^{2+}/\text{C}_0\) IOH-NPs can be typically retrieved from urine on a time scale of 2–4 days.

To verify the dissolution of the IOH-NPs, we have initiated several test reactions with suitable model compounds. A first example relates to \([\text{ZrO}]^{2+}/[\text{UFP}]^{2-}\) IOH-NPs (UFP: umbelliferone phosphate).\cite{96} \([\text{ZrO}]^{2+}/[\text{UFP}]^{2-}\) exhibits a typical particle size of \(47 \pm 9 \text{ nm}\) and shows characteristic, but weak blue emission \((\lambda_{\text{exc}} = 455 \text{ nm})\) of UFP upon UV excitation \((\lambda_{\text{exc}} = 366 \text{ nm})\). Although less interesting for imaging purposes (see 2.2), \([\text{ZrO}]^{2+}/[\text{UFP}]^{2-}\) is very interesting since the emission intensity of free umbelliferone (UF) in solution is considerably higher compared to the solid IOH-NPs (in suspension). Thus, UF release from the IOH-NPs and thereupon, their dissolution can be directly monitored by fluorescence spectroscopy.\cite{96} Whereas the UF release in aqueous suspensions at \(pH 7\) and 37 °C is very slow, it can be significantly accelerated upon addition of acid phosphatase (Figure 21). Accordingly, the emission intensity rises continuously after phosphatase addition over a period of 10 h indicating the release of UF from the \([\text{ZrO}]^{2+}/[\text{UFP}]^{2-}\) IOH-NPs via hydrolytic cleavage of the P–O–C ester bond (Figure 21). On the one hand, this verifies the dissolution of the IOH-NPs, and on the other hand, this specific reaction can also serve as a fluorescent probe of the presence of phosphatases.\cite{96} To classify the timescale of the release reaction, it must be noticed that the hydrolysis of P–O–C ester bonds is particularly fast if the phosphate group is directly linked to an aromatic system P–O–C$_\text{aromatic}$.\cite{78} In the case of aliphatic systems P–O–C$_\text{aliphatic}$, the release is significantly slower and moves on a timescale of several days.\cite{98}

A second example to illustrate the dissolution of the IOH-NPs is \([\text{ZrO}]^{2+}/[\text{AAP}]^{2-}\), containing the analgetic prodrug acetaminophen phosphate (AAP).\cite{98} Here, the dissolution of the IOH-NPs and the release of acetaminophen (AA) were evaluated based on two different approaches. First, the carbon content of \([\text{ZrO}]^{2+}/[\text{AAP}]^{2-}\) was determined by EA (Figure 22a). Second, the fluorescence of mixed-anion \([\text{ZrO}]^{2+}/[\text{AAP}]_{0.9}[\text{UFP}]_{0.1}\) IOH-NPs containing 90 mol-% of [AAP]$^{2-}$ and 10 mol-% of fluorescent [UFP]$^{2-}$ was monitored.\cite{98} Whereas EA is indicative for the AAP-related carbon content in the residual solid IOH-NPs, the fluorescence intensity refers to the released amounts of AA and UF in the solution. During the experiments, the IOH-NPs were continuously stirred in aqueous HEPES buffer at neutral pH and 25 °C. After certain periods of time, a defined aliquot of the suspension was extracted and centrifuged to obtain the IOH-
NPs and to perform EA. [ZrO]$_2^+$ [AAP]$_{2}$ [UFP]$_{2}$ was treated similarly with repetitive measurements of the emission intensity (Figure 22b). Both measures – determination of carbon content and of emission intensity – indicate a continuous release of AA and UF with the as-expected exponential slope. Over 18 and 48 h, a total release of about 60 and 80% was observed, respectively. All in all, the release data stemming from carbon content and fluorescence detection show good coincidence.

Again, the timescale of release is comparably fast, since both AAP and UFP exhibit direct bonding of the phosphate group to an aromatic system (P–O–C$_{aromatic}$). [99]

3.4. Drug Release and Delivery

Slow dissolution of IOH-NPs is not only relevant in terms of biocompatibility and biodegradability, but it is also the key to transfer the IOH-NP concept to drug release and drug delivery. Hence, the material platform and the feasibility of the IOH-NPs can become even broader. [ZrO]$_2^+$ [AAP]$_{2}$, containing the analgetic prodrug acetaminophen phosphate, can be considered as a very first example for drug delivery and release that combines uncomplex synthesis in water with very high drug load of 68 wt-% AAP. [99]

Actually, the material concept has been already expanded to drug delivery and drug release, especially in the case of phosphate-based IOH-NPs. In this regard, numerous phosphate-functionalized pharmaceutical agents are available. Similar to the fluorescent [ZrO]$_2^+$ [R$_{phosphate}$]$_2$ IOH-NPs, a general composition [ZrO]$_2^+$ [R$_{phosphate}$]$_2$ can be derived for drug-containing IOH-NPs with a phosphate-functionalized pharmaceutical anion [R$_{phosphate}$]$_2$. [99] Illustrative examples comprise, for instance, [ZrO]$_2^+$ [FdUMP]$_2$ and [ZrO]$_2^+$ [CLP]$_2$ that contain the cytostatic agent 5'-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP). [100] The anti-inflammatory agent betamethasone phosphate (BMP), [35,101] and the antibiotic agent clindamycin phosphate (CLP) (Figure 23). [102] Similar to fluorescent IOH-NPs, [ZrO]$_2^+$ [R$_{phosphate}$]$_2$ also show excellent uptake into cells at high biocompatibility. [100-102] In difference to [ZrO]$_2^+$ [UFP]$_2$ and [ZrO]$_2^+$ [AAP]$_{2}$ (see 3.3), the phosphate group in [ZrO]$_2^+$ [FdUMP]$_2$ and [ZrO]$_2^+$ [BMP]$_2$ is linked to an aliphatic system (P–O–C$_{aromatic}$) that only shows slow hydrolysis and drug release (i.e. 5–10% release of total drug content after 48 h). [101,102]

The anti-proliferative potential of [ZrO]$_2^+$ [FdUMP]$_2$ (shown with 75 wt-% load of active FdUMP) was exemplarily shown on human mammary carcinoma cells and compared to non-active [ZrO]$_2^+$ [UMP]$_2$ IOH-NPs (in suspension) as negative control (UMP: uridine monophosphate) as well as to the clinically applied 5-FU (in solution) as positive control (5-FU: 5-fluorouracil). [100] Whereas [ZrO]$_2^+$ [UMP]$_2$ (negative control) had no effect on the cell viability, 5-FU (positive control) and [ZrO]$_2^+$ [FdUMP]$_2$ IOH-NPs show considerable cytostatic effects. Interestingly, the anti-proliferative activity of the [ZrO]$_2^+$ [FdUMP]$_2$ IOH-NPs is even higher than of 5-FU (positive control), although applied with identical concentration of the active agent. [100] [ZrO]$_2^+$ [BMP]$_2$ – as the second example – contains 81 wt-% of the glucocorticoid BMP and shows excellent anti-inflammatory response in vitro (MHS macrophages, primary mouse macrophages, human peripheral blood monocytes). [99,101] In vivo studies, furthermore, indicate a promising therapeutic efficiency in a mouse model of multiple sclerosis with a strongly increased cell-type specificity for macrophages compared to conventional free glucocorticoids (in solution). [102] [ZrO]$_2^+$ [CLP]$_2$ IOH-NPs, finally, represent a novel nanoparticle-based strategy to treat persisting and recurrent Staphylococcus aureus-caused infections. [ZrO]$_2^+$ [CLP]$_2$ also contains an extremely high amount of 82 wt-% of the clinically approved antibotic clindamycin phosphate and shows high uptake at low toxicity. In comparison to the free drug in solution, most interestingly, the [ZrO]$_2^+$ [CLP]$_2$ IOH-NPs (in suspension) result in a 70 to 150-times higher drug uptake into cells, although both – free drug and IOH-NPs – were administered in identical concentrations.

Besides [ZrO]$_2^+$ [FdUMP]$_2$, [ZrO]$_2^+$ [BMP]$_2$ and [ZrO]$_2^+$ [CLP]$_2$, and their application for tumors, inflammation and infection, we could realize phosphate-based [ZrO]$_2^+$ [R$_{phosphate}$]$_2$ IOH-NPs with about 50 different pharmaceutical agents, which illustrates the feasibility of the concept as a general platform of materials. Drug delivery and drug release, however, are not a subject of this review and therefore only conceptually discussed as an additional option. Similar to [ZrO]$_2^+$ [HPO$_4$]$_2$ (see 2.2), the pharmaceutical anion [R$_{phosphate}$]$_2$ can be also partially exchanged by a fluorescent dye anion [R$_{dye}$]$_2$. In order to maintain maximum drug load, the fluorescent dye anion is available only in low concentrations of 0.005 to 0.05 mol·%. Specifically, this results in [ZrO]$_2^+$ [FDUMP]$_{0.95(ICG)0.05}$, [ZrO]$_2^+$ [BMP]$_{0.95(FMN)0.05}$ or [ZrO]$_2^+$ [CLP]$_{0.995(DUT)0.005}$, which show drug release and which can be also detected via their fluorescence (see 2.2, 2.3, 3.1, Figure 23). [100-102]

Figure 23. IOH-NPs for drug release with fluorescence labelling exemplarily shown for: a) [ZrO]$_2^+$ [FdUMP]$_2$ [ICG]$_2$, b) [ZrO]$_2^+$ [BMP]$_2$ [FMN]$_2$, and c) [ZrO]$_2^+$ [CLP]$_2$ [DUT]$_2$ (modified reproduction from ref. [100-102]).
4. Conclusions

Phosphate- and sulfonate-based IOH-NPs with a general composition \([\text{ZrO}_2\cdot x(\text{AAP})]^{4-}\cdot n(\text{AAP})\cdot (\text{TFPP})^{4-}\cdot x(\text{AAP})\cdot (\text{AAP})^+\cdot x(\text{TPPS}_4)^3-\cdot x(\text{AAP})\] exhibit several features that differ from alternative fluorescent nanomaterials, including: (i) Straightforward aqueous synthesis; (ii) Good water solubility; (iii) Exceptionally high load of fluorescent dye (70–85 wt% per nanoparticle); (iv) Use of many approved fluorophores; (v) High biocompatibility and high biodegradability. All these aspects are highly relevant to medicine and clinical practice. In addition to full-color emission, the IOH-NPs can feature even more functionalities. With Gd\(^{3+}\) as the inorganic cation, for instance, the IOH-NPs are magnetic and suitable for MRI. IOH-NPs such as \([\text{GdO}_2\cdot \text{ICG}]^+\) are multimodal and applicable for IL (due to the emission of ICG), PAI (due to the absorption of ICG) and MRI (due to the paramagnetism of Gd\(^{3+}\)). Moreover, IOH-NPs like Gd\(^{3+}\) \([\text{AAPCS}_4]^4-\) or La\(^{3+}\) \([\text{TPPS}_4]^3-\) show photo-induced ROS generation (singlet oxygen) and become suitable for photodynamic therapy. Finally, the phosphate- and/or sulfonate-based fluorescent anion can be replaced by pharmaceutical anions to realize IOH-NPs showing drug delivery and drug release. All these different features can be available in a single IOH-NP by combining two or more functional organic anions. In all, the combination of inorganic cations and functional organic anions, like from a construction kit, allows realizing multimodal and multifunctional IOH-NPs with many more compositions and functions, which, in fact, is the most relevant feature and advantage of the IOH-NPs.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: inorganic-organic hybrids · phosphates · sulfonates · fluorescence · imaging


a) A. Reisch, A. S. Klymchenko, Small 2012, 16, 968.

a) V. Sharma, P. Tiwari, S. M. Mohin, Nanoscale 2015, 7, 6904.


a) A. Reisch, A. S. Klymchenko, Small 2016, 12, 13674;

c) M. J. Schermerhorn, Nature 2017, 551, 176;

a) A. Reisch, A. S. Klymchenko, Small 2012, 16, 4286;
c) N. Zhang, E. Ding, X. F. Sun, Q. Liu, C. J. Li, Coatings 2018, 8, 133;


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FOCUS REVIEW

Extremely high dye contents (70–85 wt-%) are available in inorganic-organic hybrid nanoparticles (IOH-NPs) such as [ZrO]^{2+} [RDye_{OPO_3}]^{2-}. They show intense emission, high photostability, high cell uptake at low toxicity, and they are also suitable for multimodal imaging, singlet-oxygen generation and drug delivery.