From corrosion casting to virtual dissection: contrast-enhanced vascular imaging using hafnium oxide nanocrystals

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Abstract
Vascular corrosion casting is a method used to visualize the three dimensional anatomy and branching pattern of blood vessels, guiding insight into health and cardiovascular disease pathogenesis and progression. A polymer resin is injected in the vascular system and, after curing, the surrounding tissue is removed. This corrosion process often deforms or even fractures the fragile cast, resulting in an overall loss of information. Here, we propose a method that does not require corrosion of the tissue, based on in-situ high-resolution computed tomography (micro-CT) scans. Since there is a lack of CT contrast between the polymer cast and the animals’ surrounding soft tissue, we introduce hafnium oxide nanocrystals (HfO$_2$ NCs) as CT contrast agents into the resin. The NCs dramatically improve the overall CT contrast of the cast and allow for straightforward segmentation in the CT scans. We designed the NC surface chemistry to ensure colloidal stability of the NCs in the casting resin, resulting in a homogeneous dispersion that remains stable during casting and curing. Using only 5 m% of HfO$_2$ NCs, high-quality casts of both zebrafish and mouse models could be segmented using CT imaging software, allowing us to differentiate even µm scale details, without having to alter the resin injection method or affecting the resin’s mechanical properties. Our new method of virtual dissection by visualizing casts in-situ using contrast enhanced CT imaging greatly expands the application potential of the technique.

Introduction

Cardiovascular diseases are currently the leading cause of death worldwide.$^1$ New strategies to battle disorders affecting this system are continuously in development but require an in-depth understanding of disease progression and pathogenesis pathways. The usage of (non-)mammalian animal models has strongly advanced our knowledge of the complex cardiovascular system, aiding research and development of new treatments.$^{2-4}$ An important prerequisite is the ability to visualize the vascular tree in three dimensions throughout the
developmental stages of the model organisms, both on a macro and a micro scale. The latter in particular is highly challenging to visualize using standard techniques for cardiovascular phenotyping such as \textit{in vivo} ultrasound imaging\textsuperscript{5,6} or histological analysis.\textsuperscript{7} As an alternative approach, vascular corrosion casting has become an established method for visualization of cardiovascular morphology.\textsuperscript{8–14} During corrosion casting a liquid casting resin, typically methylmethacrylate-, polyurethane- or epoxy-based,\textsuperscript{15–18} is first injected intravenously shortly after euthanizing the animal. The resin is then allowed to cure and harden inside the vascular system, after which the soft tissue is removed via chemical maceration (corrosion) in a potassium hydroxide (KOH) solution. The cast creates a negative replica of the cardiovascular system which can then be analyzed using conventional techniques such as light microscopy, high resolution micro-computed tomography (micro-CT), and scanning electron microscopy (SEM) to capture relevant information ranging from the macro to the micro scale. While often used for resolving the vasculature, corrosion casting can also be used for other applications, such as visualization of ductal systems and pocketing structures.\textsuperscript{19}

Although corrosion casting has been shown to be a powerful technique to visualize vascular structures in model systems such as mice,\textsuperscript{20–23} rats,\textsuperscript{24,25} rabbits\textsuperscript{26–28} and zebrafish,\textsuperscript{3,6,8} it has several important shortcomings. One of the issues is the fragility of the produced cast.\textsuperscript{15,19,29} During the consecutive corrosion, rinsing and drying steps, the brittle cast is prone to breaking, resulting in a loss of information particularly when using high-resolution techniques as the finest vascular capillaries will be among the first to crack. To circumvent these issues, \textit{in-situ} micro-CT scanning of the cast has been a topic of great interest as omitting the corrosion step causes the cast to remain perfectly intact while surrounded by soft tissue. However, as CT uses differences in X-ray attenuation to generate a tomographic image, it is extremely difficult to distinguish the cast from its surrounding soft tissue via segmentation, i.e. virtual dissection. Segmentation is a post-processing technique which categorizes different materials in a scan by their difference in grayscale value, i.e. their contrast
value, therefore these grayscale values need to be sufficiently different for this technique to successfully distinguish each material. While in-vivo contrast enhanced CT imaging has been proposed as an alternative, this approach has many downsides compared to the visualization of a vascular cast using CT. In-vivo CT scans cannot reach the desired high resolution to visualize the complete vascular microstructure (≤20 µm). Additionally, they are limited to the allowed radiation dose and in the amount of contrast agent that can be injected. Finally, typical preclinical contrast agents have the tendency to accumulate in the liver and are highly challenging to inject in-vivo in smaller animals such as zebrafish. Similarly, while ex-vivo injection of contrast agents, without introduction of a polymer cast in the vascular system, has been done, this approach is prone to suffer from macroscopic and microscopic changes to the vasculature after the animal has been euthanized. Besides this, there is a need for visualization of combined casts as well, where there are two or more intertwined systems that need to be distinguished from each other. Here the addition of a contrast agent to one and not the other would make it possible to do segmentation and visually separate one part from the other. Efforts have already been made to introduce high-density dopants to casting resins, typical commercially available materials such as barium sulphate or bismuth oxide nanoparticles, however stable nanocomposite mixtures were not obtained. The instability of the contrast agents in the resin lead to agglomeration and/or gravitational sinking during the curing process, even after they were brought into a homogeneous suspension by heavy stirring. This causes local artefacts to become visible in micro-CT instead of creating homogeneous contrast enhancement, therefore making semi-automatic segmentation difficult to impossible and ultimately resulting in a loss of information that can be obtained from these scans.

The combination of polymers with nanofillers (structures that have at least one dimension below 100 nm) is referred to as polymer nanocomposites. Though research has been done on nanosheets and nanotubes, recent nanocomposites focus mainly on nanoparticles.
(NPs) as filler due to their large interface. They have a positive effect on the mechanical, thermal, electrical, optical or even fire-retardant properties of the polymers. The manufacturing approach of the nanocomposite has a large effect on its final properties. To this day the main challenge remains the prevention of agglomeration of the NPs. There are three ways to make NP-based polymer nanocomposites: (i) generating the particles \textit{in-situ}, (ii) direct mixing of NPs with polymers and (iii) \textit{in-situ} polymerization where the particles are mixed with the monomer resin, followed by polymerization of the mixture. The first option of generating the particles \textit{in-situ} has been employed for SiO$_2$, TiO$_2$ and metal such as Au and Pd. The precursors are mixed in with the polymers under appropriate conditions and when particles are formed they are therefore immediately coated by the polymer. The main drawback of this approach is the limited flexibility in possible synthesis and processing conditions. Direct mixing on the other hand is popular due to its simplicity, often done by mixing methods such as mechanical agitation, ultrasonic vibration, shear mixing, etc. to ensure a proper dispersion. For instance Al$_2$O$_3$ particles and ZnS:Mn particles have been homogeneously dispersed this way by thoroughly mixing them with an MMA monomer and then adding the initiator and chain reagent. For in-situ polymerization, it is important that the nanoparticles are properly dispersed before the polymerization process starts. Mixing the particles alone is not sufficient to prevent agglomeration for smaller (< 20 nm) particles, which have an increased surface energy, which favors agglomeration. Issues with stability in the monomer mixture or polymer can be traced back to a lack of control concerning particle size distribution and colloidal stability. While originally the surface chemistry of particles was overlooked, focus has shifted more towards ligand engineering of NPs. Ligand design for a certain application depends on the polymer matrix, as well as the nature of the NP used. Great colloidal stability has been obtained by using strongly bound matrix-compatible surface ligands. Matching the ligand to the monomer mixture or polymer improves the enthalpic compatibility. In the case of \textit{in-situ} polymerization it is also an option to incorporate ligands with end functionalities that can copolymerize with
the monomers. This covalent linkage is established via the *grafting to* method, where the NP surface functional groups can react directly with the functional groups of the polymer, or via the *grafting from* method, where the polymer chain growth is initiated on the NP surface.\(^{52,55}\) Typical examples of reactive groups are methacrylate groups\(^ {56,57}\) or epoxy groups.\(^ {58}\) The use of *click chemistry* has become a popular tool to introduce these reactive groups due to its flexibility.\(^ {58,59}\) The design in surface chemistry can range from single populations which are compatible to the monomer to complex multimodal ligands depending on the need.\(^ {52}\) Specifically when larger mass fractions are needed, (multimodal) polymer brushes have been applied, where the ligand itself is a polymer.\(^ {60-63}\) The choice of ligand complexity depends on the matrix' molecular weight as well, as the entropic penalty of the polymer to penetrate the brush increases with increasing molecular weight.\(^ {52}\)

For CT contrast enhancement, high-atomic number (Z) NPs that will strongly attenuate the X-ray beam are necessary to create the desired contrast with surrounding tissue. NP-based CT contrast agents are rapidly gaining popularity in clinical settings,\(^ {64,65}\) with examples such as Au,\(^ {66,67}\) TaO\(_x\),\(^ {68,69}\) WO\(_x\),\(^ {70,71}\) Bi\(_2\)S\(_3\),\(^ {72,73}\) and HfO\(_2\). However their use in polymer nanocomposites has been virtually nonexistent.\(^ {74}\) Hafnium oxide nanocrystals (HfO\(_2\) NCs) are an interesting candidate to improve CT contrast due to the high density and atomic number of Hf and the chemical stability of HfO\(_2\). They have been proven to effectively increase CT contrast,\(^ {75-78}\) outperforming popular current contrast agents such as iodine and gold at tube potentials of 80 kV and above.\(^ {79,80}\)

In this study, we present a new method to visualize cardiovascular casts using HfO\(_2\) NCs as contrast agents. Starting from a fast, low-cost and gram-scale synthesis of < 10 nm HfO\(_2\) NCs, we optimize the surface chemistry to provide a homogeneous dispersion in Mercox II, a commercially available casting resin. After demonstrating that the particles remain stable during curing and effectively improve the CT contrast of these nanocomposites, we inject
both zebrafish and mouse models with the NC-doped resin and obtain high-quality cast visualization via segmentation without having to adapt the existing injection methods. Our results emphasize the application potential these type of contrast agents have in corrosion casting, while paving the way to obtain full cardiovascular information from casts without needing tissue corrosion.

Results and discussion

HfO$_2$ nanocrystal synthesis

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  synthesized aggregated HfO$_2$ nanocrystals (NCs) from hafnium chloride and benzyl alcohol in a solvothermal process, taking three days. The process can be shortened to 3 - 4 hours by microwave radiation, and the resulting particles can be de-aggregated using a mixture of fatty acid and an amine. One problem with the method is the dissolution of hafnium chloride in benzyl alcohol. The metal chloride reacts exothermically with benzyl alcohol, leading to decomposition of the precursor into an insoluble solid. This precluded upscaling of the method to gram scale reactions, though the reproducibility was somewhat improved by using benzyl ether as co-solvent.

To lower the reactivity of hafnium chloride, we first synthesized its complex with tetrahydrofuran. We scaled up the original procedure of Manzer et al. to 30 grams per synthesis (with a chemical yield of 93%). The HfCl$_4$.2THF complex has the cis octahedral stereochemistry. It dissolves readily and reproducible in benzyl alcohol, eliminating the need for co-solvents. The concentrations could be increased up to 0.25 M with the THF complex. The mixture of HfCl$_4$.2THF in benzyl alcohol is subjected to microwave heating, first 5 min at 80 °C to ensure all precursor is completely dissolved, followed by the reaction step of 4 hours at 220 °C (figure 1A). These synthetic optimizations enabled a scaled-up synthesis, producing multiple grams of HfO$_2$ NCs in a single run, with a chemical yield up to 89 %. The resulting
HfO$_2$ NCs are ellipsoidal in shape and have a major axis of 6.2 ± 4.8 nm and a minor axis of 4.0 ± 2.4 nm ($\mu$ ± 3$\sigma$) as determined by transmission electron microscopy (TEM) (figure 1B). They possess the monoclinic (P2$_1$/c) crystal structure, evidenced by powder X-ray diffraction (XRD) analysis (figure 1C). The HfO$_2$ surface is functionalized with oleic acid, an inexpensive ligand which provides excellent colloidal stability in nonpolar solvent such as chloroform. The functionalization is performed in the presence of an alkylamine base to neutralize the surface adsorbed HCl on the as-synthesized particles.$^{83}$ The particles are extensively purified by precipitation and redispersion cycles to remove any unbound ligand and the alkylammonium chloride salt. The $^1$H NMR spectrum of the oleate functionalized
NCs (figure 1E) shows broadened peaks, indicating bound oleate, as peak broadening is a typical attribute of bound ligands and is caused by both heterogeneous line broadening (correlated to incomplete ligand shell solvation) and homogeneous line broadening (due to fast T2 relaxation). The NC solution has a Z-average value of 17 ± 6 nm (μ ± 3σ) in chloroform according to dynamic light scattering (DLS) which means particles are stabilized well in the solvent and there is only little agglomeration (figure 1D). Due to the voxel sizes of μCT, which are in the μm range, this residual aggregation will not be visible. Thermogravimetric analysis (TGA) shows a mass loss of 15 m% (figure S1), corresponding to a ligand density of 2.7 ligands/nm² at the nanocrystal surface (calculations in Supporting Info).

**Tuning the nanocrystal surface chemistry**

To visualize the vascular casts using contrast enhanced CT, the HfO2 NCs should increase the overall contrast of the resin without being a detectable phase themselves. As high-resolution (micro) CT can obtain resolutions in the sub-micrometer range,87,88 the NCs should remain stable and largely agglomeration-free throughout the casting and hardening of the resin. We chose the Mercox II resin for casting, a methyl methacrylate-based resin that is commonly used in corrosion casting due to its low viscosity (0.56 mPa.s at 20 °C), which allows it to fill even the finest capillaries.15,19,89,90 Here, it is used in combination with Batson’s #17 catalyst (initiator) diluted in acetone, a benzoyl peroxide catalyst which initializes the radical polymerization into poly-methyl methacrylate (PMMA). This formulation causes the resin to start curing approximately 10 minutes after mixing in the BPO catalyst and will be fully cured after 20 to 30 minutes.

Given that the HfO2 NCs are capped with oleate (HfO2-oleate) after synthesis, we used this as a model system by mixing in 5 m% of HfO2 (ligand weight subtracted), with the resin and measuring this mixture using DLS. 5 m% was chosen as an initial starting point to obtain sufficient contrast enhancement. Due to the resins’ low viscosity, it is compatibly
with DLS and allows for direct monitoring of the colloidal stability of the NCs in the resin. The volume distribution of the HfO$_2$-oleate NCs in the resin is shown in figure S2-3, where a broad peak indicates a high degree of agglomeration. The transparent resin also becomes highly turbid upon mixing in the NCs. The strong mismatch in polarity between the oleate-capped NCs and the MMA-resin indicates that surface ligands with a better compatibility need to be found. The affinity of the ligand to the resin is determined both by its head group which binds to the NC surface and its organic tail which is directed outwards into the solvent and therefore carries the most importance in terms of colloidal stability and solvent compatibility. A matching ligand tail for a given solvent has negative chain-solvent mixing energy, therefore minimizing the chain-to-chain interactions which cause NCs to agglomerate. We explored a series of ligands on the NC surface (figure 2A), varying head groups (catechol, carboxylic acid, bisphosphonic acid and phosphonic acid) as well as ligand tails. We used both polar ligands composed of ethylene glycol segments that have proven to provide excellent stability in a broad range of solvents,$^{86,91-97}$ as well as nonpolar ligands which can copolymerize with the monomer resin. All ligands were exchanged starting from HfO$_2$-oleate. (Bis)phosphonic acid binding groups are known to have a higher binding affinity for the metal oxide NC surface compared to carboxylates,$^{91,98-104}$ which allows them to be exchanged in a quantitative ligand exchange process. Carboxylic acids on the other hand can only exchange oleate when added in excess as it is an equilibrium reaction. Catechols were recently proven to provide high colloidal stability to metal oxide NCs in aqueous environments and can quantitatively displace carboxylic acids in these solvents.$^{105}$ Figure S3 shows the different ligands bound to the NC surface as evidenced by the broadened $^1$H NMR peaks.

A complete overview of all DLS measurements are shown in figure S2, those that were visually too turbid were not further characterized. Of the remaining candidates, two showed promising results: (i) a carboxylic acid, 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (MEEAA) and (ii) a phosphonic acid, (6-[2-2-(2-methoxyethoxy)ethoxy]ethoxyhexyl)phosphonic acid.
PA-hex-mPEG). The DLS data, shown in figure 2B, shows that the NCs largely retain their colloidal stability within the resin mixture. For MEEAA, a main peak at 137 ± 21 nm (90 v%) and a smaller agglomerated peak at 849 ± 127 nm (10 v%) can be observed in the volume size distribution plot. PA-hex-mPEG has its mean peak at 163 ± 41 nm (92 v%), as well as a smaller peak at 5120 ± 795 nm. This confirms that the versatility of the PEG segment provides greater colloidal stability in the resin compared to the purely aliphatic chain of other ligands tested. The NCs functionalized with the nonpolar ligands methylmethacrylic acid and undecelynic acid that can copolymerize with the resin could not be dispersed homogeneously.

![Figure 2](image_url)

Figure 2: (A) Overview of the different ligands tested, classified by binding group and polarity of the ligand chain. (B) Volume size distribution DLS data of 5 m% HfO₂ NCs (ligand weight subtracted) in the casting resin, functionalized with MEEAA (left) and PA-hex-mPEG (right).

Surprisingly, the bisphosphonic acid does not provide the same stability as PA-hex-mPEG to the NCs. Immediately after mixing the resin became visually very turbid, rendering it
impossible to get accurate DLS results. We hypothesize that this is due to the lower ligand
density, which we calculated to be 1.3 ligands/nm\(^2\) based on TGA results, compared to 2.7
ligands/nm\(^2\) for PA-hex-mPEG (figure S1). The stability of catechols in the resin is not so
straightforward because they are known to have weaker binding in nonpolar solvents, but
give excellent stability in aqueous environments. However, we found that upon mixing the
nitrodopamine-mPEG stabilized NCs with the resin, it immediately turned turbid. A mixed
ligand shell of phosphonic acids was also tested, as these are known to provide even higher
stability than ligand shells containing only a single ligand.\(^{106}\) We therefore made a ligand
shell composed of 50% PA-hex-mPEG and 50% 2-ethylhexyl phosphonic acid. The latter,
a so-called 'entropic ligand', has been proven to be more dispersible than long, straight
chains.\(^{91,107}\) While a minimum amount of visual turbidity was observed after mixing, DLS
analysis indicated a higher fraction of agglomeration than for the purely PA-hex-mPEG
functionalized NCs (figure S2). We hypothesize that the non-polar nature of the ligand has
a greater negative effect than the entropic gain. Finally it was determined if the method of
mixing the HfO\(_2\) NCs with the resin influences their colloidal stability. Thus far, all tests
were performed through addition of the NCs in the resin as a powder. We hypothesized that
by already having the ligand shells solvated, the NCs would be less prone to agglomerate
upon mixing with the resin. To test this, HfO\(_2\) NCs capped with PA-hex-mPEG were
suspended in a minimal volume of acetone, the same solvent the benzoyl peroxide catalyst is
dissolved in, and added to the resin. DLS results showed that adding the NCs in suspension
did not further improve their colloidal stability, additionally we found that removal of the
extra acetone added proved to be difficult without negatively affecting the resin. From our
preliminary experiments we thus concluded that ligand shells composed of 100% MEEAA
or PA-hex-mPEG mixed in powder form to the resin would give the best results in terms of
colloidal stability.
Preliminary micro-CT visualisation

To further confirm that the NCs themselves are not visible as a heterogeneous mixture in micro-CT scans, we made samples of HfO$_2$ NCs capped with MEEAA and with PA-hex-mPEG mixed in the resin with 5 m\% (ligand weight subtracted) as our initial concentration, pending further optimization in the next stage of our experiments. After addition of the catalyst, the samples are fully cured after 10 to 20 minutes. Small fragments were cut from these hardened samples and analyzed via micro-CT, where they were scanned at a peak tube potential of 80 kV and a 7$^3$ $\mu$m$^3$ voxel size to get high resolution. The tube potential in CT determines the quality and quantity of the photons generated and an increase in kV extends and intensifies the X-ray spectrum, such that both the maximal and the average (effective) energy are higher.$^{108}$ The choice of tube potential affects the CT contrast and tube potentials of $\geq$ 80 kV result in better contrast with HfO$_2$ due to its favorably located K-edge at 65 keV. Figure 3 shows that doping the resin with 5 m\% of HfO$_2$ NCs indeed increases the grayscale values of the samples compared to an undoped reference. This preliminary result already indicates that the contrast between the cast and the surrounding tissue will be effectively improved when injecting the doped resin \textit{ex vivo}. However, the CT image of the sample containing NCs functionalized with MEEAA is littered with white dots. These grayscale hotspots imply that despite the stability of MEEAA in the casting resin (figure 2B), the NCs start to agglomerate during the curing process and to the point where they are larger than the CT voxel size. We hypothesize that the ligands are stripped from the NC surface during curing, causing NC agglomeration. PA-hex-mPEG does not have this issue due to its higher binding affinity for the NC surface.$^{103,109}$ Indeed, throughout the entire volume of the micro-CT scan of the PA-hex-mPEG capped NC-containing sample no dots can be found. Even though DLS analysis indicated some initial agglomeration, they do not agglomerate further during curing and therefore remain beneath the detection limit of micro-CT, making it the ideal candidate for injection. A volume render of the entire sample can be found in the supporting information (figure S5), showing that no agglomerations can be found throughout
the complete sample volume.

Figure 3: High-resolution CT scan of samples made with, from left to right, 0 m% HfO₂ (reference), 5 m% (ligand weight subtracted) HfO₂ capped with MEEAA and with PA-hex-mPEG.

**Optimizing the post-synthetic surface modification**

After establishing that PA-hex-mPEG gives colloidal stability in the resin before and during curing, we sought to make the exchange process more straightforward. Since the synthesis starts from the HfCl₄·2THF precursor, chlorides and protons are adsorbed on the NC surface after the reaction (HfO₂·H⁺Cl⁻). In a typical post-synthetic surface modification for this synthesis, the mixture is brought into a nonpolar environment and the chloride anions are replaced by carboxylates, e.g. oleate, in the presence of an alkylamine. Charge neutrality is required in nonpolar solvents, therefore the exchange equilibrium is determined by ligand binding enthalpy, steric, solubility and the proton transfer. The large pKa difference between oleic acid (pKa ≈ 5.02) and hydrogen chloride (pKa ≈ -8) makes this proton transfer the driving force between the exchange, but the addition of a base such as oleylamine reverses this equilibrium and allows oleate to bind to the surface due to its now higher binding affinity. The amount of oleate on the HfO₂ NC surface is then determined via ERETIC measurements and the particles are exchanged via a quantitative X-for-X ligand exchange to the phosphonic acid (figure 4A-B), followed by a second purification round. PA-hex-mPEG has a pKa₁ ≈ 2.99 and pKa₂ ≈ 8.33 and therefore also needs a base to replace the chloride in nonpolar solvents. A direct exchange from HfO₂·H⁺Cl⁻ to the phosphonic acid becomes
difficult due to the ligand’s second acidic proton which can react with the base and create a salt that precipitates.

Figure 4: (A) Ligand exchange from chloride to oleate to PA-hex-mPEG. (B) $^1$H NMR spectra of the NCs capped with oleate (purple) and after ligand exchange to PA-hex-mPEG (green), all in CDCl$_3$. The inset shows the $^{31}$P NMR spectrum after the exchange. (C) Direct exchange from chloride to PA-hex-mPEG. (D) $^1$H NMR spectra of the NCs before and after purification. The inset shows the $^{31}$P NMR after purification.

The need for proton transfer can be circumvented by working in polar solvents, where the strict requirement of charge neutrality is no longer present. After synthesis, the HfO$_2$.H$^+$Cl$^-$ NCs are dispersed in an aqueous phase, which is diluted with ethanol. We use a photometric chloride test to calculate the amount of chloride adsorbed on the NC surface, taking into account variations in HfO$_2$ NC yield (calculations in the Supporting Info). Addition of PA-hex-mPEG to this mixture results in broadened $^1$H and $^{31}$P NMR peaks, indicative of bound
phosphonates. Yet, the detected chloride concentration was higher than with the indirect exchange from chloride to oleate to phosphonate, the latter leaving almost no trace of chloride. These observations indicate that PA-hex-mPEG only partly exchanges the chlorides, creating a mixed shell of chloride and PA-hex-mPEG on the NC surface. Addition of triethylamine (Et$_3$N) drives the exchange equilibrium forward and allows the PA-hex-mPEG to replace the remaining chlorides adsorbed on the surface. Purification by precipitation/redispersion cycles removes the Et$_3$N$^+$.Cl$^-$ and excess Et$_3$N and phosphonic acid (figure 4C-D). After purification, no chloride is detected anymore on the NC surface. TGA confirms that the ligand density is comparable to the indirect exchange from oleate to phosphonate, validating the full exchange to PA-hex-mPEG without the need for a carboxylate in-between step.

**Cardiac injection using HfO$_2$ NC-doped casting resin and high-resolution CT scan**

Having demonstrated that our HfO$_2$ NCs effectively improve the CT contrast of the resin, the NC-doped resin was tested *in vivo* to demonstrate its feasibility of vascular casting. We opted for two animal models for our proof-of-concept: adult zebrafish and mice. Zebrafish are interesting subjects for vascular corrosion casting as they are well-suited for the study of genetic diseases and are deemed complementary to mammalian animal models, such as mice. Nevertheless, their small size poses technical challenges for resin injection and maintaining cast integrity. Mice have been used extensively as established animal models for a range of disorders, also often after genetic modification. The mixture is prepared by weighing the appropriate amount of PA-hex-mPEG-functionalized HfO$_2$ NCs in powder form and thoroughly mixing them with the casting resin, followed by addition of the catalyst. No viscosity increase is observed upon mixing the NCs with the resin, allowing for the casting procedure to proceed identically as with an undoped resin.

Cardiac injection in small animals is inherently a very precise and difficult technique,
meaning that independent of injection with a NC-doped or undoped resin mixture not all samples will be successful. Preliminary radiographic sample screening can allow a fast and straightforward first qualitative control of the cast and promising samples can then be scanned using micro-CT at the desired resolution. Figure S7 shows that the complete process of reconstruction, segmentation and subsequent 3D rendering is not necessary when the only interest is check if casting was executed successfully. From the CT slices alone, which are by definition radiographs, one can see the cast and its contrast difference with the surrounding soft tissue, making it clear that we can proceed with a micro-CT scan. Alternatively, a fast low-resolution overview scan can also reveal the success of casting, after which a higher-resolution scan of the region-of-interest is possible. Promising NC-doped samples were scanned in micro-CT. We opted for high-resolution scans where we reduce the voxel size as much as possible while still having the complete animal in the field-of-view. When interested in specific regions, e.g. the kidneys of a mouse or the gills of a zebrafish, it is possible to zoom-in on this region alone to achieve even higher resolutions. Samples were scanned by removing them from the ethanol or water solution used for storage, placing them in a plastic container and scanning them open to air.

Zebrafish were scanned using micro-CT with a voxel size of \(3.5^3 \mu m^3\) which allows to visualize the complete fine structure of the cast. The NC-doped polymer cast can be clearly distinguished from the soft-tissue (figure 5A). Two scans were performed on the same fish: one before and one after bone decalcification. Due to the calcium present in bones, which has a higher atomic number compared to the soft tissue, they are clearly visible in CT. Decalcification selectively removes these calcium salts, but does not affect the CT contrast between the soft tissue and the cast, therefore simplifying the segmentation to separate the cast from the soft tissue. Segmentation is a semi-automated technique which discriminates voxels based on their grayscale value. We propose this technique of virtual dissection as an alternative to the corrosion casting process. It was possible to fully segment the cast showing
Figure 5: Micro-CT data of zebrafish casted with 5 m% HfO$_2$ NCs doped in the resin. (A) Before decalcifying (left), the segmented cast is given a red color for clear distinction from the bones, with next to it the cardiovascular scan that was segmented after a second scan at higher resolution after decalcifying. Several parts of the cardiovascular casts are coloured and assigned. (B) Synchrotron CT scan slices of the zebrafish’ gills as shown from the XY-, YZ- and XZ-plane.

even the smallest veins and gills using the region growing approach, which was previously impossible without the addition of the HfO$_2$ NCs. Region growing was chosen due to its high accuracy and ease in use. It starts from a manually placed seed, continuously evaluating neighbouring voxels grayscales and growing towards voxels of high similarity to distinguish one phase from another.

Zebrafish were also scanned using synchrotron X-ray tomography. Synchrotron-based sources can achieve high resolutions using phase-contrast, but contrast is not sufficient to segment the cardiovascular structure.$^{110}$ Using the NC-doped resin, the contrast is remarkably improved.
Figure 5B shows slices from different points of view of a scan focusing on the zebrafish’ gills, visualizing high levels of detail. Some agglomerations could be distinguished, though it was possible to segment the complete cast (figure S8).

To show the applicability of our technique on larger animals, we repeated the injection and CT scan procedure on mice. Figure 6 shows two different mice, injected with 5 m% NC-doped resin and scanned with a voxel size of $20^3 \mu m^3$. We opted for bigger voxel sizes compare to the smaller zebrafish samples in order to have the complete vascular structure in the field-of-views. The figure shows the 3D volume render of the mice, visualizing all the fine structure, as well as the segmented cardiovascular structure with special focus on the abdominal region, as it is often of interest. The casts were again segmented using region growing, making it possible to segment the cast from the rest of the mouse. A zoom of the kidneys reveals their fine structure.

Figure 6: Micro-CT data of mice casted with 5 m% HfO$_2$ NCs doped in the resin. (A) 3D volume render of the mice shows all the fine structure revealed by the cast. (B) Segmented cast (in red) with mouse bone structure for reference. In the top picture the aorta is also segmented. (C) Zoom-in on the kidney region of the segmented cast to show the level of detail in the micro-CT scan.
Conclusion

We have presented a new method to visualize cardiovascular casts by using contrast enhanced computed tomography, making using of virtual dissection, i.e. in-situ analysis, by using HfO$_2$ NCs as CT contrast agents. The NCs can be synthesized on gram-scale using affordable precursors. We have demonstrated the importance of optimizing the surface chemistry to obtain a homogeneous dispersion of the NCs in the resin that remains stable during curing and have presented a suitable phosphonate ligand which strongly binds to the NC surface. The phosphonate can now be exchanged on the NC surface in a single-step post-synthetic modification. With only 5 m% of NCs mixed in the resin, CT contrast was dramatically improved and we were able to segment high-quality casts from high-resolution CT scans of both zebrafish and mice injected with the NC-doped resin. This method can easily be expanded to other models besides zebrafish and mice, effectively eliminating the need for corrosion from vascular corrosion casting. By retaining the tissue, this method also allows for future correlative microscopy methods in which microscopic analysis of the surrounding tissue can be correlated with the local three dimensional structure of the blood vessels.

Experimental

Materials

Hafnium(IV) chloride (98%), Oleic acid (90%), Oleylamine (70%), 10-Undecenoic acid (98%), Methacrylic acid (99%), Triethylamine ($\geq$99.5%) and Chloroform (stabilized with amylenes, $\geq$99%) were purchased from Sigma-Aldrich. Benzyl alcohol was either purchased anhydrous (99.8%) or as ReagentPlus® ($\geq$99%) from Sigma-Aldrich, the latter was then vacuum distilled and stored over sieves. Tetrahydrofuran (ExtraDry, 99.5%) was purchased from Acros Organics. 2-[2-(2-Methoxyethoxy)ethoxy]acetic acid ($>95.0\%$) was purchased from TCI Chemicals. 6-[2-2-(2-methoxyethoxy)ethoxy]ethoxyhexyl)phosphonic acid (PA-hex-mPEG)
(96%) was purchased from Sikémia. (2-2-[2-(2-2-[2-(2-methoxyethoxy)ethoxy]ethoxy)ethoxy]ethoxy)bisphosphonic acid (bisPA-mPEG) was purchased from Surfactis Technologies. N-(4,5-dihydroxy-2-nitrophenethyl)-2-[2-(2-methoxyethoxy)ethoxy]acetamide (NDA-mPEG) was synthesized according to Deblock et al.\textsuperscript{105} 2-Ethylhexyl phosphonic acid (EHPA) was synthesized according to De Roo et al.\textsuperscript{91} Mercox II resin was purchased from LADD research industries. Batson’s #17 Catalyst was purchased from Polysciences. Solvents used for synthesis were purchased from Chemlab or Sigma-Aldrich. All deuterated solvents were purchased from Sigma-Aldrich or Eurisotop. All manipulations are performed in air unless otherwise indicated. All chemicals are used as received unless otherwise mentioned. When required, organic solvents are dried according to the procedure described by Williams et al.\textsuperscript{112} making use of 20% m/v freshly activated 3Å sieves for a minimum of 120 h.

**HfCl\textsubscript{4}.2THF precursor synthesis**

The procedure was adapted from Manzer et al.\textsuperscript{84} In a nitrogen-filled glovebox, 22 g of HfCl\textsubscript{4} (1 eq, 0.069 mol) is added to 220 mL anhydrous DCM, only partly dissolving. Next, 22 mL (3.95 eq, 0.271 mol, 19.56 g) of anhydrous THF is added in a dropwise manner and under vigorous stirring. The HfCl\textsubscript{4} dissolves completely while adding THF and a few minutes after complete addition, crystals start precipitating. The solution is placed in the freezer (-18 °C) for 1 hour and then filtered over a por 4 filter funnel. The resulting powder is washed with 110 mL dry pentane, resulting in a free flowing white powder. Another 220 mL dry pentane is added to the motherliquor and a second fraction precipitates. The solution is placed in the freezer for another hour, after which it is filtered and washed again with 110 mL dry pentane. Both fractions are combined and dried overnight under vacuum. The product is characterized by FTIR and \textsuperscript{1}H NMR, shown in figure S9.
Microwave-assisted solvothermal synthesis of HfO$_2$ nanocrystals and post-synthetic surface modification with oleate

The microwave-assisted synthesis is based on the original solvothermal synthesis by Buha et al.,$^{81}$ which was later adapted into a microwave-assisted synthesis by De Roo et al.$^{82}$ Microwave procedures were conducted using a CEM Discover SP with autosampler and a CEM Mars 6 using the EasyPrep Plus vessels. Both microwaves operate at a frequency of 2.45 GHZ.

Small-scale synthesis

Precursor preparation is executed in a nitrogen-filled glovebox. Under vigorous stirring, 4 mL (38 eq, 38 mmol, 4.16 g) of anhydrous benzyl alcohol is added to 0.464 g (1 eq, 1 mmol) of HfCl$_4$.2THF in a 10 mL microwave vial with stirring bar. The solution is removed from the glovebox and stirred for 5 minutes, resulting in a transparent solution. The mixture is subjected to microwave heating for 5 minutes at 80 $^\circ$C (30 W), followed by 4 hours at 220 $^\circ$C (300 W) at medium stirring and PowerMax off. After synthesis, the mixture is transferred to a 15 mL plastic centrifuge tube using a Pasteur pipette. The microwave vial is rinsed with 3 mL of diethyl ether, in order to maximize the yield, after which this is also added to the centrifuge tube. After mild centrifugation (720 rcf, 2 min), three phases are observed: a transparent organic (top) phase, an aqueous, milky (middle) phase and sometimes a solid (bottom) phase of insolubles. If the work-up is done the same day as the synthesis, the solid phase is usually avoided. The transparent (top) phase is removed and the milky phase is separated from the solid phase using a Pasteur pipette in a separate plastic centrifuge tube. The solid phase is discarded. Ethanol is added to the milky phase, yielding 2 mL of translucent suspension. 5 mL of diethyl ether is added and the particles are precipitated (4500 rcf, 2 min), resulting in HfO$_2$ nanocrystals capped with HCl. The particles are redispersed by sonication in 1 mL of chloroform and 150 $\mu$L (0.47 mmol, 0.134 g) of oleic acid is added to the milky suspension. Next, 125 $\mu$L (0.38 mmol, 0.101 g) of oleylamine is
added, instantly resulting in a transparent suspension. The particles are purified by adding 5 mL of acetone, followed by centrifugation (4500 rcf, 4 min), removal of the organic top phase, and resuspension in 1 mL of chloroform. This purification step was repeated three times. Sonication can be used to help redisperse the particles in chloroform.

Large-scale synthesis

Precursor preparation is executed in a nitrogen-filled glovebox. 40 mL anhydrous benzyl alcohol (38 eq, 0.38 mol, 41.6 g) is added to the liner of the EasyPrep Plus vessel. 4.65 g HfCl$_4$·2THF (1 eq, 0.01 mol) is carefully added to the liner under vigorous stirring. The edges of the liner are rinsed with the solution to remove any powder stuck to the edge. Two liners are prepared simultaneously. The solution is stirred for 5 minutes, resulting in a transparent solution. The mixtures are subjected to microwave heating for 5 minutes at 80 °C (10 minute ramp, 200W), followed by 4 hours at 220 °C (20 minute ramp, 800W). Figure S10 shows the microwave profile of the temperature and pressure over time of a typical reaction. After synthesis, the mixture is split evenly over two centrifuge tubes. The liner is rinsed with 30 mL diethyl ether, which is also split over the two centrifuge tubes. After mild centrifugation (720 rcf, 2 min), the transparent top phase is removed and 10 mL of ethanol is added to each milky phase, yielding a translucent suspension. 25 mL diethyl ether is added to each tube and the particles are precipitated (4500 rcf, 4 min). The particles are redispersed by sonication in 5 mL of chloroform and 750 µL (2.36 mmol, 0.668 g) of oleic acid is added to the milky suspension. Next, 625 µL (1.89 mmol, 0.506 g) of oleylamine is added and the solution is sonicated for 5 minutes, resulting in a transparent suspension (a slight Tyndall effect is normal). The particles are purified by adding 15 mL of acetone, followed by centrifugation (4500 rcf, 4 min), removal of the organic top phase, and resuspension in 3 mL of chloroform. This purification step was repeated three times. Sonication can be used to help redisperse the particles.
Ligand exchange from oleate

Ligand exchange from oleate to the phosphonic acids (PA-hex-mPEG and PA-hex-mPEG/2-ethylhexyl phosphonic acid (EHPA) mixed shell) and bisphosphonic acid (bisPA-mPEG) was done as follows:

The amount of oleate surface ligand was determined via the digital ERETIC method\textsuperscript{113} based on the alkene resonance located around 5.3 ppm and the methyl resonance located around 0.9 ppm in deuterated chloroform for $^1$H NMR measurements. For PA-hex-mPEG, 1.2 equivalents were added to the nanocrystal solution using a 100 mM stock solution in chloroform. For bisPA-mPEG, 0.5 equivalents were added to the nanocrystal solution using a 100 mM stock solution in chloroform. The mixture was subjected to sonication for 15 minutes. The nanocrystals were precipitated by addition of hexane (mixture of isomers), typically in a 1:5 volume ratio. After centrifugation (4500 rcf, 4 min), the particles were redispersed in a minimal volume of chloroform. This purification procedure was repeated three times. Sonication can be used to help redisperse the particles.

For the PA-hex-mPEG/EHPA mixed shell, 0.5 equivalents of EHPA were added to the nanocrystal solution using a 100 mM stock solution in chloroform, followed by sonication for 60 minutes at 40 °C. The nanocrystals were precipitated by addition of acetone, typically in a 1:5 volume ratio. After centrifugation (4500 rcf, 4 min), the particles were redispersed in 1 mL of chloroform. Next, 0.5 equivalents of PA-hex-mPEG were added to the nanocrystal solution using a 100 mM stock solution in chloroform, followed by sonication for 30 minutes. The particles were purified two more times by addition of hexane (mixture of isomers), typically in a 1:5 volume ratio, centrifugation (4500 rcf, 4 min) and redispersion in a minimal volume of chloroform. Sonication can be used to help redisperse the particles.

Ligand exchange from oleate to the carboxylic acids (MEEAA, methacrylic acid and 10-undecenoic acid) was done by determining the amount of oleate surface ligand as described above. 2 equivalents of carboxylic were added to the nanocrystal solution, followed by 30
minutes of sonication. The nanocrystals were precipitated by addition of hexane (mixture of isomers) for MEEAA and methacrylic acid and acetone for 10-undecenoic acid, typically in a 1:5 volume ratio. After centrifugation (4500 rcf, 4 min), the particles are redispersed in a minimal volume of chloroform and another 2 equivalents of carboxylic were added. This procedure is repeated three times in total. For the last step, the purification procedure was repeated three times. Sonication can be used to help redisperse the particles.

For the catechol (NDA-mPEG), nanocrystals were exchanged from oleate to MEEAA and subsequently exchanged to NDA-mPEG as described by Deblock et al.\textsuperscript{105}

**Ligand exchange from chloride to phosphonic acid**

Direct exchange after synthesis to PA-hex-mPEG was done as follows:

After synthesis, the particles are precipitated with diethyl ether and redispersed in 2 mL of ethanol as described above. 300 µmol of PA-hex-mPEG is added to the particles using a 500 mM stock solution in ethanol, followed by 250 µmol of triethylamine using a 1M stock solution in ethanol. The mixture was subjected to sonication for 30 minutes after which the mixture is transparent. The nanocrystals are precipitated by addition of diethyl ether and hexane, typically in a 1:0.5:5 volume ratio. After centrifugation (4500 rcf, 4 min), the particles were redispersed in a minimal volume of chloroform. This purification procedure was repeated two more times, but by redispersing the particles in ethanol. Sonication can be used to help redisperse the particles.

**Resin mixture preparation and cardiac injection**

The mixture is prepared by allowing the monomer and catalyst, stored at 4 °C, to get to room temperature. 5 m% of HfO$_2$ NCs are weighed, taking into account the density of Mercox (0.95 g mL$^{-1}$) and the ligand weight (17 m%, figure S1). The monomer resin is added to this powder and mixed thoroughly. The particles have long-term stability both in solution and stored as a powder, their stability has been checked the same day, after a
month and after three months and did not change over time (figure S11). It is advised to mix the NCs in with the resin right before injection, as the NCs do not provide a long-term stability in the monomer resin and will start to agglomerate over time. Typically 1 mL of resin is prepared for injection in zebrafish and 2 mL for injection in mice, corresponding to respectively 60.25 mg HfO$_2$ NCs and 120.5 mg HfO$_2$ NCs stabilized with PA-hex-mPEG. 200 µL of catalyst is added per mL of resin. After mixing in the catalyst, the NC-doped resin is injected. The resin starts curing after 10 minutes and is fully cured after 20 - 30 minutes.

**Injection of zebrafish**

The HfO$_2$/polymer mixture is injected as described previously by De Spiegelaere *et al.*

Briefly, zebrafish are euthanized, and after immobilization the heart is exposed via an incision. Glass needles (borosilicate glass capillary tubes, O.D. 1.5 mm, I.D. 1.10 mm; Sutter Instrument), are filled with the resin and connected to a microinjection system (FemtoJet 4i; Eppendorf) for delivery of small volumes. With the aid of a Leica M80 stereomicroscope (Leica Microsystems), the needle is inserted into the ventricle parallel to the bulbus arteriosus at a 45° angle, and injection is started using a continuous flow. Initially, a constant pressure of 10 hPa is used, which is gradually increased over an injection time of approximately 10-15 minutes until the flow stops due to increased resistance by the hardening resin. Throughout the injection procedure, the minimal pressure allowing flow into the heart is used to avoid deformation and damage to the vascular walls. The total injection volume varies between 10-20 µl. After careful removal of the needle, the specimen is fixed in Formaldehyde 4% (VWR Chemicals) for at least 24h. To minimize interference of mineralized bone tissue during micro-CT imaging, the zebrafish are decalcified using a Citric Acid solution containing 45% Formic acid and 20% Sodium citrate for 4 to 6 hours. The decalcified specimen is then preserved in 70% Ethanol.
**CT scan postprocessing**

Datasets were processed using the VGStudio Max software. CT scans were imported as an image stack. For segmenting bigger structures the 3D median filter was applied first with filter size: \( x = 3, y = 3 \) and \( z = 3 \) which reduces noise by replacing each voxel’s grayscale value by the median of the surrounding voxel’s grayscale value. The cast was segmented from the surrounding tissue by creating a selection using the semi-automatic region growing (flooding) algorithm. A seed point was manually placed in any of the 2D views after which the algorithm grows the selection, continuously expanding as long as the neighbouring voxels are within the user-specified tolerance relative to the seed. The region growing algorithm was used in static mode, i.e. voxels are added to the selection if they are connected to the region and if their grayvalue does not deviate by more than half the chosen tolerance from the grayvalue of the seed. No other constraints (such as max. radius) where applied to algorithm. The tolerance value and seed point were changed until an optimal selection was obtained. For some data, the Opening/Closing mode is applied, which eliminates gaps in the selection that was grown by expanding the selection with the user-specified number of voxels and then immediately contracting. Finally, the selection is extracted from the volume as a separate region-of-interest (ROI). The 3D volume render was exported as an image as isosurface render or as scatter HQ render. The isosurface volume render displays the surface of the selected ROI as defined by the manually set iso-level. The scatter HQ volume render is used for the visualization of faint grayvalue differences and surface structure details.

**TEM analysis**

High-resolution transmission electron microscopy (HRTEM) was performed on a JEOL JEM-2200FS TEM with Cs corrector operating at 200 kV. Samples were made by drop-cast suspension on the grids. The TEM grids used were Holey-Carbon - Cu (C200-CU) with 50 micron hole size (200 mesh). NC sizes were determined by measuring 200 particles using the ‘polygon selection’ tool of ImageJ, with measurements set to ‘fit ellipse’.
**XRD analysis**

X-ray diffraction (XRD) was performed on a Bruker D8 Advance with motorized anti-scatter screen and Autochanger and Bragg-Brentano $\theta-\theta$ geometry (goniometer radius 280 mm). The instrument uses the Cu Kα radiation ($\lambda = 1.54184 \ \text{Å}$) with no Kβ filter. The detector is a LynxEye XE-T Silicon strip Line detector with 192 channels. Samples were made by drop-cast suspension on a glass plate. The measurement was performed in the $2\theta$ 15 - 60° range at a step size of 0.02° and a scan rate of 0.5°/min.

**DLS analysis**

Dynamic Light Scattering (DLS) measurements were recorded on a Malvern Zetasizer ZS instrument in backscattering mode (173°) at a temperature of 20 °C. Measurements were performed in glass cuvettes and ran 3 to 6 times after equilibrating for 60 seconds. For the Mercox II resin as dispersant, the dispersant refractive index was set as 1.141 and the viscosity as 0.56 cP. DLS data processing was performed using the Malvern ‘ZS Explorer’ software using the ‘general purpose’ analysis model.

**NMR analysis**

Nuclear magnetic resonance (NMR) spectra were recorded at 298K on a Bruker 300 instrument at 400 MHz, a Bruker Avance III spectrometer operating at a $^1$H frequency of 500.13 MHz and featuring a BBI probe and a Bruker UltraShield 500 spectrometer operating at a $^1$H frequency of 500.13 MHz. Chemical shifts (δ) are given in parts per million (ppm) and the residual solvent peak was used as an internal standard (CDCl$_3$: $\delta$H = 7.24 ppm, MeOD: $\delta$H = 3.31 ppm (quintet). Chemical shifts for $^{31}$P spectra were referenced indirectly to the $^1$H NMR frequency of the sample with the xiref-macro in Bruker. For the quantitative 1D $^1$H measurements, 64k data points were sampled with the spectral width set to 16 ppm and a relaxation delay of 30s. Quantification was done using the Digital ERETIC method.$^{113}$
**TGA analysis**

Thermogravimetric analysis (TGA) was done with a Netzch STA 449F3 Jupiter instrument. Powder samples were heated from 25°C to 1200°C at 10K/min under air. A correction file was recorded using the same conditions. TGA data processing was performed using the Netzch Proteus Analysis software.

**UV-Vis analysis**

UV-Vis spectra were recorded on a PerkinElmer Lambda 365 measuring the absorbance (A) from 500 to 400 nm with a slit width of 1 nm and scan speed of 240 nm/min.

**FTIR analysis**

Fourier-Transform Infrared Spectroscopy (FTIR) was performed on a PerkinElmer spectrum 2 ATR-FTIR with a diamond crystal measuring 8 scans from 450 to 4000 cm⁻¹ and using background subtraction.

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**Supporting Information Available**

The Supporting Info is available.
References


(38) de Oliveira, A. D.; Beatrice, C. A. G. Polymer nanocomposites with different types of nanofiller. *Nanocomposites-Recent Evolutions* 2018, 103–104.


(49) Siegel, R.; Chang, S.; Ash, B.; Stone, J.; Ajayan, P.; Doremus, R.; Schadler, L. Me-


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