Mapping out the aqueous surface chemistry of metal oxide nanocrystals; carboxylate, phosphonate and catecholate ligands

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Abstract

Iron oxide and hafnium oxide nanocrystals are two of the few successful examples of inorganic nanocrystals used in a clinical setting. Although crucial to their application, their aqueous surface chemistry is not fully understood. The literature contains conflicting reports regarding the optimum binding group. To alleviate these inconsistencies, we set out to systematically investigate the interaction of carboxylic acids, phosphonic acids and catechols to metal oxide nanocrystals in polar media. Using Nuclear Magnetic Resonance spectroscopy and Dynamic Light Scattering, we map out the pH-dependent binding affinity of the ligands towards hafnium oxide nanocrystals (an NMR compatible model system). Carboxylic acids easily desorb in water from the surface and only provide limited colloidal stability from pH 2 – 6. Phosphonic acids on the other hand provide colloidal stability over a broader pH range but also feature a pH-dependent desorption from the surface. They are most suited for acidic to neutral environments (pH < 8). Finally, nitrocatechol derivatives provide a tightly bound ligand shell and colloidal stability at physiological and basic pH (6-10). While dynamically bound ligands (carboxylates and phosphonates) do not provide colloidal stability in phosphate buffered saline, the tightly bound nitrocatechols provide long term stability. We thus shed light on the complex ligand binding dynamics on metal oxide nanocrystals in aqueous environments. Finally, we provide a practical colloidal stability map, guiding researchers to rationally design ligands for their desired application.

Introduction

Colloidal nanocrystals (NCs) have been considered for a multitude of biomedical applications, e.g., bio-imaging,¹⁻³ drug delivery,^{4, 5} photothermal therapy,⁶ and radiotherapy enhancement.^{7, 8} These NCs are typically hybrid objects, consisting of an inorganic core capped with organic ligands. Ligands determine the interactions between NC and solvent and the stability of the nanocolloid.⁹ In the case of biomedical applications, controlling the NC surface chemistry is key since it will play a role in particle agglomeration, cellular uptake,¹⁰⁻¹² protein repelling or adsorption,^{11, 13, 14} cytotoxicity,^{11, 15} circulation time^{14, 16} and targeted approaches.¹⁷⁻¹⁹ While surface chemistry is important for all types of NCs (chalcogenides, pnictides, halides, and metal NCs), not one solution fits all. For example, thiolates and thiols have a strong binding affinity to Au and CdSe NCs but interact poorly with metal oxide NCs.²⁰

Metal oxide NCs have been particularly successful in nanomedicine. Three types of inorganic NCs have achieved clinical translation and two of them are oxides; iron oxide and hafnium oxide.⁵ These particles are often first synthesized in nonpolar solvents, stabilized by surfactants (usually with a carboxylate or phosphonate head group, and an aliphatic tail). Carboxylic acids (e.g., oleic acid) dissociate on the metal oxide surface, with carboxylates binding to surface metals sites and protons to surface oxygen atoms.²¹⁻²³ This binding motif is written as NC(XX') since both proton and carboxylate are X-type ligands.²⁴ In nonpolar solvents, carboxylic acids are quantitatively exchanged by phosphonic acids in an X-for-X ligand exchange process.²⁵ Indeed, phosphonic acids are very strong ligands for oxide surfaces.^{26, 27} In contrast, catechol was found to be a rather weak ligand, only able to exchange a minor fraction of oleic acid.²⁸ There is thus a clear order in binding strength in nonpolar solvents: catechol < carboxylic acid < phosphonic acid. In aqueous (or other polar) environments, the order is less clear, and further complicated by variable factors such as pH and salt concentration. For example, carboxylic acids are frequently used to stabilize metal oxide NCs in water.²⁹⁻³³ They are able to provide colloidal stability in static systems with no competing ligands, but not in phosphate buffered saline (PBS) or cell culture media.³³ The binding affinity is significantly increased for multidentate carboxylate ligands such as polymers.^{34, 35} In general, literature

reports agree that carboxylic acids are the weakest ligands in water, weaker than phosphonic acids or catechols.³⁶ The literature is however inconclusive as to whether phosphonic acids or catechols are the best ligand. For example, Guénin et al. concluded from FTIR and density functional theory (DFT) calculations that caffeic acid (a catechol) has a higher affinity for 9 nm iron oxide NCs than a bisphosphonate, at physiological pH.³⁷ In contrast, Zeininger et al. determined that phosphonic acids bind more strongly than catechols to TiO₂ NCs, in isopropanol at pH 7.³⁸ Okada et al. came to the same conclusion on 9 nm iron oxide NCs by performing phase-transfer experiments using polar and nonpolar phosphonic acids and catechols.³⁹ It is clear that despite the common usage of phosphonic acids and catechols in metal oxide NC functionalization, there is no clear consensus on the relative binding affinity. Furthermore, a direct link between ligand binding equilibria and the final colloidal stability of the NCs is usually not made.

In this paper, we aim to unambiguously establish the binding affinity order and provide the correct surface chemistry for optimal application in nanomedicine. We chose HfO₂ NCs as our model system for two reasons: (1) it is a relevant material in nanomedicine, and (2) it is compatible with solution Nuclear Magnetic Resonance (NMR) spectroscopy. The latter has proven to be a very powerful tool to study nanocrystal surface chemistry.⁴⁰⁻⁴⁴ Unfortunately, iron oxide NCs interfere with magnetic fields and cannot be studied in NMR. HfO₂ NCs are thus an ideal starting point, also because their surface chemistry has already been extensively studied in nonpolar solvents using NMR spectroscopy.^{21, 22} First, we evaluate the ligand exchange of the native carboxylic acid ligands for phosphonic acid and catechol ligands, using ¹H and ³¹P NMR spectroscopy. Importantly, we use the same polyethyleneglycol ligand chain for all three binding groups, thus ensuring that we can directly compare the binding groups to each other. Next, we assess the influence of solvent (methanol vs water) and the pH on ligand binding. Furthermore, we used NMR and Dynamic Light Scattering (DLS) to determine the colloidal stability provided by the different ligand types in aqueous and buffer environments, and directly correlate this to ligand binding dynamics. Finally, we constructed a colloidal stability map, showing which binding group provides colloidal stability as a function of pH. This practical guide will help researchers in designing future surface chemistries.

Results

The HfO₂-MEEAA model system. We synthesized HfO₂ nanocrystals (NCs) from hafnium *tert*-butoxide and benzyl alcohol at 220 °C via an established solvothermal process (Figure 1A).⁴⁵ The HfO₂ surface was functionalized with 2-[2-(2-methoxyethoxy)ethoxy]acetic acid (MEEAA, see Figure 1B) to stabilize the nanocrystals in toluene and all unbound ligands were removed as described previously.⁴⁴ The nanocrystals have a diameter of 2.64 ± 0.19 nm ($\mu \pm \sigma$) according to TEM (Figure 1C) and possess the monoclinic (P2₁/c) crystal structure according to X-ray total scattering and pair distribution function (PDF) analysis (Figure S1). The ¹H NMR spectrum of the nanocrystal dispersion in toluene-*d*₈ only shows broadened resonances, assigned to bound ligands (Figure 1B). Indeed, spectral broadening is a typical attribute of bound ligands due to both homogeneous broadening (T₂ relaxation) and heterogeneous broadening (imperfect solvation of the ligand shell).⁴⁴

Figure 1. (A) Solvothermal synthesis of HfO₂ nanocrystals starting from 1 equivalent Hf(O-*t*Bu)₄ and 88 equivalents benzyl alcohol. (B) ¹H NMR spectra (normal or diffusion filtered) of MEEAA functionalized HfO₂ NCs in different solvents. The **α** and **β** resonances belong to the residual hydroxyl and methyl groups of methanol, respectively. (C) Transmission Electron Microscopy (TEM) image of the synthesized HfO₂ NCs. The NC diameter of the quasi-spherical NCs was calculated after

measuring the surface area of at least 150 NCs and calculated the diameter as if it was a circle. A size distribution histogram and a zoomed-in image of a singular NC can be seen respectively in the bottom left and the top right corner.

The nanocrystals can also be dispersed in ethanol, methanol and water, and are thus an ideal starting point for our investigation into ligand binding behaviour in polar solvents. In methanol- d_4 (MeOD) the ^1H NMR spectrum looks markedly different with sharp signals superimposed on the broad resonances (Figure 1B). We assign the sharp signals to auto-desorbed ligands, corroborated by the appearance of two sets of resonances in Diffusion Ordered Spectroscopy (DOSY, Figure S2). DOSY allows to separate (overlapping) NMR resonances according to their diffusion coefficient and thus separates the free ligand (quickly diffusing) from ligands bound to NCs (slowly diffusing).⁴⁶ In Figure S2 the resonances with a diffusion coefficient $D = 95 \text{ m}^2/\text{s}$ represent the bound ligands and the resonances with $D = 450 \text{ m}^2/\text{s}$ belong to the free ligands. We can selectively observe the bound ligands in a diffusion filtered spectrum (Figure 1B), and upon close inspection, one observes that the bound MEEAA resonances in methanol- d_4 are slightly sharper than the ones in toluene- d_8 indicating a better solvation of the ligand shell in methanol.⁴⁴ Given that all ligands were bound in toluene, we infer that the solvent clearly plays a role in the adsorption-desorption equilibrium, for instance by changing the solubility of the ligand.⁴⁷ Indeed, even a higher fraction of MEEAA ligands desorbs in water, D_2O (see Figure S3), but the nanocrystals remain stable in the pH range 2 – 6. The NCs quickly precipitate at $\text{pH} > 6$ and therefore MEEAA is not a suitable ligand for many biomedical applications, which typically require stability at physiological conditions ($\text{pH} = 7.4$).

Note that aside from the main MEEAA signals, we also observed broad resonances with low intensity in the aromatic region of the ^1H spectrum (Figure S4), assigned to benzoate ligands. Benzoic acid was previously identified as a side-product of the nanocrystal synthesis and found adsorbed on the nanocrystal surface.⁴⁵ Functionalization of the surface with MEEAA after synthesis clearly did not remove all benzoate from the surface and a small fraction remains present.

Competitive binding of phosphonic acids. To evaluate the binding strength of phosphonic acids in methanol, we chose (2-(2-(2-hydroxyethoxy)ethoxy)ethyl)phosphonic acid (PA-PEG) as a ligand with comparable structure to MEEAA, (Figure 2A). In the ^1H NMR spectrum, resonance **1** of PA-PEG has a chemical shift of 2.05 ppm, clearly separated from the MEEAA resonances, allowing us to selectively monitor the binding of PA-PEG (Figure 2B). Since MEEAA has a methoxy group and PA-PEG has not, resonance **f** is used to gain selective information about the binding of MEEAA. The other resonances (**2-6** and **b-e**) overlap.

Figure 2. (A) Ligand exchange performed between MEEAA functionalized NCs and PA-PEG. (B) ^1H NMR reference spectra in MeOD of the free ligands as reference and the stepwise titration of MEEAA functionalized NCs with PA-PEG, equivalents are with respect to the total amount of MEEAA present. (C) ^{31}P NMR spectra (4096 scans) in MeOD for the stepwise titration of MEEAA functionalized NCs with PA-PEG, broadened signals are indicative of NC binding. (D) Diffusion filtered ^1H NMR

spectra of MEEAA functionalized NCs in MeOD after addition of 1.3 equivalents of PA-PEG. Signals arising from bound MEEAA are denoted in red, signals arising from PA-PEG are denoted in striped blue. $C_{\text{NC}} = 1210 \mu\text{mol.L}^{-1}$, corresponding to 34 mg NCs of this size in 0.5 ml MeOD. Resonances denoted as * are unidentified impurities.

Starting from MEEAA stabilized HfO_2 nanocrystals in methanol- d_4 , we add PA-PEG in steps while monitoring the ^1H NMR spectrum, see Figure 1B (full range spectra and additional titration points in Figure S5). During the titration, we observe the gradual appearance of a broad resonance around 2.2 ppm, assigned to resonance **1** of bound PA-PEG. Concomitantly, resonance **f** becomes more narrow (Figure 2B), indicating the removal of MEEAA from the nanocrystal surface. Within the aromatic region we also observe desorption of benzoic acid (Figure S5). We conclude that PA-PEG is effectively displacing MEEAA and benzoic acid. Given the absence of free phosphonic acid, the exchange is quantitative for most of the titration. After addition of 1.3 equivalent of phosphonic acid, sharp signals appear in the same region, indicating that there is now free PA-PEG present. The same conclusions are drawn from the ^{31}P spectra as well (Figure 2C), where first a broad signal grows in intensity and a sharp ^{31}P signal appears after adding more than one equivalent. Interestingly, the bound PA-PEG resonance still increases slightly in intensity between 1.3 and 1.6 equivalents. We infer that the last part of the exchange does not proceed quantitatively and the remaining carboxylate ligands are more difficult to remove. This observation implies that the binding affinity (ΔG_{ads}) is not a single, fixed quantity for all the MEEAA ligands, but rather a distribution, as has been previously shown for CdSe nanocrystals.⁴⁸

Unfortunately, the NMR spectra feature a lot of spectral overlap, with superimposed signals of free and bound ligands. At the end of the titration, the resonances of free ligands dominate the spectrum. To gain more insight in the composition of the ligand shell at that point, we turn to the diffusion filtered spectrum (Figure 2D, full range in Figure S6). While resonances **2** - **6** overlap with resonances **b** - **e**, the clear presence of resonance **f** indicates the presence of residual MEEAA on the surface. Based on the diffusion filtered profile of bound MEEAA (Figure S4), we assigned the part of the spectrum belonging to MEEAA as the red shaded area. The blue patterned area is assigned to PA-PEG. There is also still benzoate present on the

surface according to the regular ^1H spectrum but the signal-to-noise of these resonances was too low in the diffusion filtered spectrum due to their faster T_2 relaxation (because of rigidity and proximity to the surface). The above results thus confirm that the exchange between the carboxylate ligands and PA-PEG does not go to completion in methanol. This conclusion stands in contrast to the relative binding affinities of fatty acids and alkylphosphonic acids in nonpolar solvents, where phosphonic acids quantitatively replace carboxylic acids in a 1:1 stoichiometry.^{25, 49, 50}

The auto-desorption of MEEAA in methanol already indicated that the ligand solubility can change the binding affinity. Indeed, ligand binding is an equilibrium process, governed by the chemical potential of each species:

Therefore, this adsorption-desorption equilibrium is dependent on the chemical potential of the free ligand (and thus the solubility).⁴⁷ To further explore this concept in practice, we designed a ligand shell architecture that would mimic a micelle; having both a hydrophobic and a hydrophilic segment. To this end we chose the ligand (6-[2-[2-(2-hydroxy-ethoxy)-ethoxy]-ethoxy]-hexyl)phosphonic acid (PA-hex-PEG), see Figure 3. We hypothesized that this ligand would more readily self-assemble on the nanocrystal surface since the hydrophobic region decreases the solubility in polar solvents. Using PA-hex-PEG, we performed the same titration experiment as before. The results are generally quite similar showing the exchange of carboxylate ligands for PA-hex-PEG (Figure S7 and Figure S8). However, this time, most benzoate ligands are removed by PA-hex-PEG (although not completely) and hardly any MEEAA resonances are detectable in the diffusion filtered spectrum (Figure 3). We conclude that indeed; the competitive binding can be manipulated by changing the ligand solubility. Interestingly, benzoic acid appears to have a higher binding affinity than MEEAA which could be ascribed to both its higher acidity and its lower solubility in methanol.

Figure 3. Diffusion filtered ^1H NMR spectrum of the NC suspension in MeOD at 1.3 equivalents PA-hex-PEG added.

Purification and transfer to water of phosphonate capped nanocrystals. With our final goal in mind of providing a stable surface chemistry for biomedical applications, we sought to purify our dispersions and disperse them in aqueous media. Precipitation-redispersion cycles are the most common way of purifying nanocrystals. However, the great versatility of the ethylene glycol segment provides colloidal stability in a broad range of solvents and non-solvents like hexane do not mix well with methanol. Therefore, we choose to purify our dispersion using spin filtration, a form of ultrafiltration. The technique is based on semipermeable membranes (like dialysis) where small molecules can pass through the pores but large nanocrystals cannot. By performing the separation in a centrifuge, purification is expedited. First, the nanocrystal suspension is placed in the spin filter and further diluted with pure methanol. Figure S9 shows that dilution does not induce ligand desorption. After filtration, a concentrated dispersion of nanocrystals is retrieved. The purification is successful as shown by the removal of almost all unbound species after 3 purification cycles (Figure S10). A comparison of the diffusion filtered spectra before and after spin filtration show a perfect match, proving that the purification did not change the ligand shell composition (Figure S10). Interesting differences between PA-PEG and PA-hex-PEG were observed when gradually changing the solvent composition from pure methanol- d_4 to D_2O (Figure 4). Whereas PA-PEG gradually desorbs from the surface when increasing water content, PA-hex-PEG remains tightly bound. This further underscores our hypothesis that PA-hex-PEG behaves as a micelle mimic, avoiding contact of the

hydrophobic segment with water. By binding the nanocrystal surface, the PA-hex-PEG ligand creates a hydrophobic inner shell with alkyl-alkyl interactions and a hydrophilic outer shell with ethylene glycol moieties as hydrogen bond acceptor for water molecules. PA-PEG on the other hand is highly water soluble and thus the affinity for the hafnium oxide surface decreases upon increasing the water content.

Figure 4. (A) and (B) ^{31}P NMR spectra of PA-PEG and PA-hex-PEG functionalized NCs at different D_2O volume %. (C) Free ligand fraction for PA-PEG and PA-hex-PEG at different D_2O volume %, determined by peak deconvolution.

Synthesis and binding of catechols. We synthesized N-(4,5-dihydroxy-2-nitrophenethyl)-2-(2-(2-methoxyethoxy)ethoxy)acetamide (nitrodopamine-mPEG), see Scheme 1. We opted for a nitrocatechol instead of an unsubstituted catechol since the nitro group decreases the catechol pKa values, and improves the oxidative stability of the catechol. Nitrodopamine was also shown to provide better colloidal stability to iron oxide nanoparticles than unsubstituted dopamine.⁵¹ Starting from dopamine hydrochloride, a one-step nitration reaction results in the formation of nitrodopamine hemisulfate. Separately, MEEAA was converted in an activated N-hydroxysuccinimide ester (MEEAA-NHS) using N,N'-dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS) and 4-Dimethylaminopyridine (DMAP). The coupling between nitrodopamine hemisulfate and MEEAA-NHS was performed using N-Methylmorpholine (NMM) acting as non-nucleophilic base. The final nitrodopamine-mPEG ligand was purified using preparative HPCL and fully characterized with ESI-HRMS and NMR spectroscopy (see Supporting Information).

Scheme 1. (A) Synthesis of nitrodopamine hemisulfate, adapted from Napolitano et al.⁵² (B) Synthesis of MEEAA-NHS, adapted from Meissler et al.⁵³ (C) Synthesis of nitrodopamine-mPEG, inspired by Amstad et al.⁵¹ Reagents and conditions: (i) NaNO_2 , 20% H_2SO_4 , H_2O , 0°C to RT, 12h, 50%; (ii) NHS, DCC, DMAP, dry THF, 0°C to RT, 12h, 83%; (iii) NMM, dry DMF, RT, 48h, 75%.

When we performed a similar competitive binding experiment as before (addition of nitrodopamine-mPEG to MEEAA capped HfO_2 nanocrystals in methanol- d_4), we found that one equivalent of nitrodopamine-mPEG was unable to effectively compete for the nanocrystal surface, even at merely 0.4 equivalents of catechol, freely diffusing nitrodopamine-mPEG signals could be observed (Figure S11). Such a low binding affinity was highly unexpected, given many reports of successful surface functionalization with catechols.^{39, 51, 54-58} However, in these reports water or biological buffers were used as the solvent. Therefore, we designed a competitive binding experiment directly in water. Fortunately, MEEAA stabilized HfO_2 nanocrystals are stable in D_2O , even though a significant portion of MEEAA is desorbed (Figure S3). The addition of one equivalent nitrodopamine-mPEG, without adjusting the pH, results in a turbid suspension with $\text{pH} = 2$. When the pH is adjusted to $\text{pH} = 5$, a stable suspension is obtained. To allow for a systematic, gradual addition of nitrodopamine-mPEG we prepared a stock solution of nitrodopamine-mPEG with 2 equivalents of NaOD to doubly deprotonate the catechol. Addition of the base changes the color of the solution from light yellow to a deep burgundy (Figure S12). We added this stock solution to a suspension of MEEAA stabilized HfO_2 , in steps of 0.5 equivalents and recorded both standard ^1H NMR and diffusion

filtered NMR spectra. After addition of 0.5 equivalents, we do not observe any sharp signals belonging to nitrodopamine-mPEG, while we do observe desorbed benzoic acid and desorbed MEEAA (Figure S13). In the diffusion filtered spectrum, we also see a clear change. In the aromatic region, the broad signals of benzoate are replaced by the broad signals of nitrodopamine-mPEG. The peak shape of the region 3-4 ppm is also altered (Figure S14). The exchange continues as more nitrodopamine-mPEG is added and upon addition of 1.5 equivalents, sharp (unbound) nitrodopamine-mPEG signals are detected (Figure S13). The suspension remains stable despite the pH being 10.3 at this point in the titration, providing further evidence that the ligand exchange was successful since MEEAA stabilized nanocrystals would precipitate at $\text{pH} > 6$. We conclude that nitrodopamine-mPEG can quantitatively displace MEEAA from the nanocrystal surface if the $\text{pH} > 5$.

The NCs were again purified using multiple cycles of spin filtration, using Milli-Q water as solvent, until the filtrate was nearly colorless (Figure S15). The concentrate was evaporated and redispersed in D_2O , and the pH was adjusted to 7.4. Figure 5B contains a regular ^1H NMR spectrum of the purified suspension, the purity of the sample is striking and only broadened resonances pertaining to nitrodopamine-mPEG are observed. Based on a TGA mass loss of 23% we calculate a nitrodopamine-mPEG ligand density of 2.14 nm^{-2} at the nanocrystal surface. We conclude that nitrodopamine-mPEG forms a tightly bound ligand shell on the nanocrystals at physiological pH with no signs of desorption.

Figure 5: (A) Ligand exchange performed between MEEAA functionalized NCs and nitrodopamine-mPEG. (B) ¹H NMR spectra before and after the ligand exchange titration performed in D₂O with nitrodopamine-mPEG. 1.5 equivalents of nitrodopamine-mPEG were added and the pH was kept above 5 at all times during addition, the purified nitrodopamine functionalized NC spectrum was measured at pH = 7.4. C_{NC} = 128 μmol.L⁻¹, corresponding to 14.4 mg NCs of this size in 2 ml D₂O.

pH-dependence of ligand binding. Since it is obvious that pH plays a crucial role in ligand binding in aqueous environments, we systematically varied the pH from 3 to 10 and used ¹H NMR, ³¹P NMR and Dynamic Light Scattering (DLS) to assess the ligand binding and colloidal stability (Figure 6). From NMR, we extract the bound ligand fraction for the phosphonic acid ligands via peak deconvolution of the ³¹P resonances and for nitrodopamine-mPEG via peak deconvolution of the aromatic ¹H resonances (Table S3-5). From DLS measurements we obtained the Z-average value and the zeta potential. The Z-average value is a single value describing the average particle size and is most sensitive to agglomeration. The zeta

potential indicates the degree of electrostatic repulsion between the nanocrystals (zeta potential values above +25 mV or below -25 mV indicate stable suspensions). From Figure 6, we clearly observe that the bound ligand fraction decreases with rising pH for both PA-PEG and PA-hex-PEG. Taking the pKa values of ethyl phosphonic acid ($pK_{a1} = 2.43$; $pK_{a2} = 8.05$) as reference, it is striking that the bound ligand fraction decreases most steeply around pK_{a2} . We infer that the second deprotonation of the phosphonic acid causes ligand-ligand repulsion in the ligand shell and increases the solubility of the ligand in water. Both effects lead to a reduced bound ligand fraction. Not surprisingly, the loss of ligands has a detrimental effect on the colloidal stability and the Z-average increases significantly for $pH > pK_{a2}$. Overall, PA-hex-PEG performs slightly better than PA-PEG but the difference is small. Most likely the double anionic phosphonate compensates for the short hydrophobic segment.

Figure 6. Effect of pH on ligand binding and stability in water for purified NCs functionalized with PA-PEG, PA-hex-PEG and Nitrodopamine-mPEG . (A) Bound and unbound ligand fraction in D₂O based on NMR peak deconvolution at different pH

values. (B) Z-average value of NCs in DLS at different pH values. (C) Zeta potential of the NCs at different pH values. All measurements were performed at constant ionic strength (0.01 mol.L⁻¹ NaCl) at 25°C.

One might expect complete loss of colloidal stability upon ligand desorption. However, no visual turbidity was observed in the samples and the Z-average remains below 100 nm. Note that the zeta potential drops below -25 mV at pH > 8. While steric stabilization is lost with progressing ligand desorption, electrostatic stabilization takes over, preventing the NCs from fully destabilizing. The negative charge could originate from residual bound, double deprotonated phosphonates, or more likely from hydroxide adsorption on the nanocrystal surface. Indeed, in water, there are multiple adsorption-desorption equilibria present simultaneously.

On top of these adsorption-desorption equilibria, there are also the acid-base equilibria, resulting in a highly complex pH dependence of the system.

According to Figure 6, the phosphonic acids keep the nanocrystals stable between pH 3 and 8 in a *static* system. However, in a dynamic biological environment (e.g. a blood vessel), many competing ligands are present and desorbed ligands are quickly removed. The equilibrium will adjust and thus ligands will continuously desorb from the surface, eventually causing loss of colloidal stability. Despite an acceptable Z-average value at physiological pH, this dynamic behaviour could explain why single phosphonate ligands are not necessarily successful at fully preventing aggregation in physiological media.⁵⁹⁻⁶³

Interestingly, nitrodopamine-mPEG has almost the complete opposite behaviour, unable to provide stable colloidal dispersions under acidic conditions with a very sharp transition around pH = 5. This is evidenced

by the steep increase in the Z-average (Figure 6). Between pH 5 and 10, all nitrodopamine-mPEG ligands remain bound and only at pH = 11, there is a slight decrease in the bound fraction from 100% to 97% (Full range ^1H NMR spectra in Figure S16). This translates in an excellent colloidal stability in pH range 5 - 11. We conclude that for aqueous applications at physiological pH, nitrodopamine-mPEG outperforms both PA-PEG and PA-hex-PEG. For aqueous applications at acidic pH on the other hand the phosphonic acid ligands are better suited. To test if there would also be a temperature dependence on the binding dynamics of the functionalized NC systems, we performed variable temperature NMR measurements in D_2O at pH 7.4 between 25°C and 60°C (Figure S17). ^1H NMR spectra showed no change in ligand adsorption/desorption equilibria for both phosphonic acids and nitrodopamine-mPEG, leading us to tentatively conclude that the results from the pH titrations would also translate to physiological temperature. To illustrate the complementary behaviour of phosphonic acids and catechols, we performed a competitive exchange reaction on purified nitrodopamine-mPEG NCs in D_2O . 1 eq of PA-PEG was added, compared to the original amount added to functionalize the NCs with nitrodopamine-mPEG, and NMR measurements were performed at different pH values (Figure S18). Figure S18 clearly shows that, at acidic pH values, a partial exchange with PA-PEG occurs, evidenced by the appearance of sharp nitrodopamine-mPEG signals in the aromatic region and methylene triplet around 3 ppm. The nitrodopamine-mPEG NC suspension, which normally would fully destabilize below pH 5, remained colloidally stable at pH 2.22 due to the now mixed catechol-phosphonate ligand shell. The exchange equilibrium mostly shifts back towards nitrodopamine-mPEG as the pH moves towards neutral and basic values.

Stability in phosphate buffered saline (PBS). Finally, we assessed the stability of NCs functionalized with PA-PEG, PA-hex-PEG and nitrodopamine-mPEG in phosphate buffered saline (PBS). Stability in PBS is an important prerequisite for biomedical applications, since many *in vivo* experiments inject the desired drug or contrast agent in saline solution or in PBS. A 1X PBS buffer contains 137 mmol.L^{-1} NaCl, 2.7 mmol.L^{-1} KCl, 10 mmol.L^{-1} Na_2HPO_4 and 1.8 mmol.L^{-1} KH_2PO_4 , and is the standard concentration. When the concentrations are half or double, these buffers are referred to as 0.5X PBS and 2X PBS

respectively. It is clear that PBS contains relatively high salt concentrations and phosphate ions (which will compete for the surface) and thus provides a real test for the stability of our functionalized nanocrystals. First, we varied the PBS concentration and immediately measured the Z-average via DLS (Figure 7A). Up to 1.5X PBS, all ligands keep the nanocrystals colloidal stable. As expected, PA-PEG is the weakest ligand and cannot prevent the start of nanocrystal aggregation in 2X PBS solutions. Second, the stability of all functionalized NCs was monitored over time in 2X PBS (Figure 7B). Clearly, PA-PEG functionalized nanocrystals agglomerate quickly and visually precipitate after several hours. The colloidal stability of PA-hex-PEG functionalized nanocrystals steadily decreases over 24 hours, before completely agglomerating. Only nitrodopamine-mPEG functionalized nanocrystals remain perfectly stable. There is no sign of aggregation over the course of 48 hours and the suspension also remains visually clear for at least 1 month.

Figure 7. Stability of functionalized NCs in different concentrations of phosphate buffered saline (PBS) at pH 7.4 and 25°C. (A) Colloidal stability of functionalized nanocrystals measured using DLS z-average values at different PBS concentrations. (B) Stability of functionalized NCs in 2X PBS over time at pH 7.4 and 25°C.

Discussion

The above results make clear that surface chemistry becomes much more complex when moving from nonpolar to polar (e.g. aqueous) solvents. In nonpolar solvents, charged ligands or nanocrystals are thermodynamically unstable, leading to a limited set of binding motifs and clear ligand exchange rules.²⁴ For example, auto-desorption of oleate (deprotonated oleic acid) does not occur in toluene. For the binding motif of $\text{PbS}(\text{PbX}_2)$, removal of the entire Lewis acid PbX_2 has been observed in chloroform or coordinating solvents like THF.⁶⁴ Likewise, for $\text{HfO}_2(\text{H},\text{OOCR})$, desorption of oleic acid is possible by recombination of the carboxylate and proton.²¹ However, these restrictions disappear in polar solvents where charges are stabilized, and the proton and carboxylate have independent adsorption/desorption equilibria, see also Equations 2-4.

From the above data, we constructed a colloidal stability map, indicating which ligands provide colloidal stability in specific pH ranges, see Figure 8. It is interesting to correlate this stability map to the pK_a 's of the ligands. We take the pK_a values of ethyl phosphonic acid ($\text{pK}_{a1} = 2.43$; $\text{pK}_{a2} = 8.05$) as reference for the PA-PEG and PA-hex-PEG ligands. At $\text{pH} = 3$ (where the particles are stable), we calculate that 96% of the phosphonic acid is mono-deprotonated. One can assume that the phosphonate will bind in this form to the nanocrystal surface. At $\text{pH} > 8$ (above pK_{a2}), the bound ligand fraction decreases quickly and the particles start to aggregate (Figure 6). The double deprotonation has two consequences; (1) the ligand becomes more soluble in water, and (2) there appears electrostatic ligand-ligand repulsion, instead of the stabilizing hydrogen bonding. Both effects promote ligand desorption. In addition, the iso-electric point of hafnia occurs at $\text{pH} = 8$,⁶⁵ rendering the surface negatively charged at $\text{pH} > 8$, thus further decreasing the binding affinity of the negatively charged phosphonate ligand. Esarey et al. observed a similar phenomenon when studying the binding constant of a phosphonate anchored rubidium complex on anatase TiO_2 surfaces under varying pH conditions.⁶⁶

Figure 8. Colloidal stability map for metal oxide nanocrystals functionalized with carboxylic acids, phosphonic acids and catechols. In this concrete example, we used HfO_2 as NC model system with its respective iso-electric point (IEP), MEEAA as carboxylic acid, PA-PEG as phosphonic acid and nitrodopamine-mPEG as catechol. The map colors should be interpreted as follows: green - excellent colloidal stability; striped green - high fraction of bound ligands but not perfect, use in a static system; orange - low bound ligand fraction, use in a static system without competition; red - nanocrystal aggregation/precipitation, do not use.

A similar reasoning applies to the case of nitrodopamine-mPEG ($\text{pK}_{\text{a}1} = 6.6$; $\text{pK}_{\text{a}2} = 11$; based on nitrodopamine pK_{a} values⁶⁷). At $\text{pH} = 5$ (where the particles are unstable), we calculate that approximately 98% of the ligand is fully protonated, and can thus only interact with the surface via weak hydrogen bonds.⁶⁸ Above $\text{pH} 5$, more nitrodopamine-mPEG becomes mono-deprotonated and is able to coordinate to the surface metal sites and provides an additional hydrogen bond to further stabilize the bound state. This is confirmed by comparing the UV-VIS spectra of nitrodopamine-mPEG bound to HfO_2 NCs with the reference spectra of the free ligand, see Figure 9 and Figure S19. For $5 < \text{pH} < 11$, we find the mono-deprotonated species. At $\text{pH} > 11$, we see again the appearance of free ligands in NMR (Figure S16) and the double deprotonated species in UV-Vis (Figure S19). For the reasons mentioned above, the double deprotonated species has a low binding affinity for the surface. In addition, the catechol becomes sensitive to oxidation at high pH. Finally, carboxylic acids can only be mono-deprotonated and we observe that NCs functionalized with MEEAA ($\text{pK}_{\text{a}} = 4.43$, experimentally determined via the Henderson-Hasselbalch equation for weak acids) gradually lose colloidal stability above $\text{pH} 3.5$ before fully precipitating above pH

= 6 (Figure S20). We hypothesize that they are less stable than phosphonates and catechols due to the lack of ligand-ligand hydrogen bonding.

Figure 9: UV-VIS spectra of purified nitrodopamine-mPEG functionalized NCs at different pH values in H₂O.

The stability experiments in buffer solution point to an additional variable: competitive ligands. In phosphate buffered saline, a high concentration of phosphate is present, which competes for the surface but does not provide steric stabilization. Since the phosphonic acids (PA-PEG and PA-hex-PEG) desorb at pH = 7.4 (25% desorbed, see Figure 6), they are in equilibrium and can thus be slowly displaced over time. The nitrodopamine-mPEG is tightly bound (0 % desorbed) and is not being displaced by phosphonic acid, judging from our competition experiments (Figure S18). Hence the high stability of the particles capped with nitrodopamine-mPEG.

The above discussion shows that in aqueous media, surface chemistry is a complex interplay between multiple factors. There is the pH-dependent charge of the surface, the pH-dependent deprotonation of the ligand, and competition by phosphate in buffer solution. Having thus the pK_a(s) of the ligands available and the isoelectric point of the nanocrystals, one can now use the colloidal stability map (Figure 8) to start predicting which ligands will provide colloidal stability at a particular pH.

Conclusion

We have mapped the binding affinity of the three most important ligands for metal oxide nanocrystal surface functionalization. By performing an in-depth surface chemistry and stability study we found that that ligand dynamics become much more complex when moving from nonpolar to polar solvents. In water, the pH influences the ligand solubility through deprotonation of the binding group. Carboxylic acids are weakly binding anchors in both nonpolar and polar environments and can be easily displaced by competitive ligands. Phosphonic acids are strongly bound ligands in MeOH but desorb in aqueous media, the extent of which is dependent on the pH. They are mostly suited to stabilize nanocrystals in acidic environments. Finally, nitrocatechol derivatives provide a tightly bound ligand shell and excellent stability at physiological and basic pH. They are superior at stabilizing metal oxide nanocrystals in phosphate buffered saline solutions, but cannot be used in acidic environments. We have summarized our results in a convenient colloidal stability map that can be generalized to other oxides and ligands, provided the isoelectric points and pka values are available. This tool will allow researchers to rationally choose nanocrystal functionalization strategies for their desired applications in polar or aqueous environments.

Associated Content

Supporting information

The Supporting Information is available free of charge at ...

Experimental methods, NMR spectra of titrations and synthesized compounds, PDF analysis, peak deconvolutions, UV-vis spectra of nitrodopamine-mPEG, DLS data of carboxylate capped nanocrystals.

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