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# The role of pH, metal ions and their hydroxides in charge reversal of protein-coated nanoparticles†

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In this study, we investigated charge inversion of protein-coated Au nanoparticles caused by the addition of metal ions. The addition of hydrolyzable metal ions (Lewis acids) can induce drastic pH changes and depending on this pH, the metal ions (e.g.  $M^{3+}$ ) are readily converted into the hydrolyzed species ( $MOH^{2+}$ ,  $M(OH)_2^+$ ) or even into hydroxides ( $M(OH)_3$ ). Adsorbed metal hydroxides were identified to cause the charge inversion of the NPs by using a combination of cryo-TEM, EFTEM and  $\zeta$ -potential measurements.

## 1. Introduction

The phenomenon of charge inversion (*i.e.* overcharging, charge reversal) has always been controversial and is still under discussion as “unambiguous experimental evidence is virtually non-existent”.<sup>1</sup> Up to now, there are several theories at hand.<sup>2</sup>

Whereas the physical approaches explain the charge inversion *e.g.* by ion–ion correlation mechanisms,<sup>1,3</sup> the chemical theories assume that a super-equimolar adsorption of oppositely charged species is the cause.<sup>1,2,4</sup>

In the case of negatively charged proteins, oppositely charged species can either be protons (pH), metal cations or metal hydroxides.<sup>1,2,5</sup> Charge inversion by pH is manifested in the isoelectrical point of proteins. What remains unclear is the origin of charge inversion when metal ions are added: it is either caused by metal ions or by metal hydroxides.<sup>1,2</sup> It is however a well-known fact that charge inversion can only be observed for hydrolyzable metal ions (mostly transition metal ions).<sup>6</sup> This means that at a specific pH the metal ion (*e.g.*  $M^{3+}$ ) converts into its hydrolyzed species ( $MOH^{2+}$ ,  $M(OH)_2^+$ ) or even its hydroxides ( $M(OH)_3$ ).<sup>7</sup> As the surface charge of these hydroxides depends on the pH as well, the pH seems to be the one key parameter that enframes all interactions.<sup>8</sup>

In literature, charge inversion is either attributed to the adsorption of unhydrolyzed ions,<sup>9–24</sup> hydrolyzed ions<sup>25–33</sup> or hydroxides.<sup>34–36</sup> Which of these species is eventually responsible,

remains unclear because the commonly used electrophoretic measurements (*e.g.*  $\zeta$ -potential measurements) alone cannot distinguish between these three species.<sup>1,2,6</sup>

Therefore, the identification and localization of the adsorbing species (the adsorbate) is crucial. To localize the adsorbate, the adsorbent (*i.e.* proteins) needs to be localized, as well. As the localization of proteins with TEM is a challenging task, immobilized bovine serum albumin (BSA) on Au NPs was applied as adsorbent in this study. Due to the high contrast of Au NPs in TEM, both, the fixed adsorbent (protein) and the adsorbate are therefore identifiable and localizable with TEM. Furthermore, the plasmonic properties of Au NPs allow it to study the influence of the metal species on the colloidal stability as the color of Au NPs changes when aggregation occurs.

## 2. Results

Au@BSA NPs were synthesized by coating citrate-stabilized Au NPs (Au@citrate) with BSA through ligand exchange, according to the method we previously reported on ref. 24 and 37–43. The average size of the Au@citrate NPs was  $15 \pm 2$  nm. The as-prepared Au@citrate NPs were added to a BSA solution (0.1% citrate, pH  $\approx$  9) at room temperature and stirred for 24 hours. The NPs were purified from protein excess and concentrated (5–10 mM Au) by fivefold centrifugation (*cf.* more details in the Experimental section). The resulting Au@BSA NPs exhibited an LSPR band, at around 525 nm, *i.e.* a red shift of 2–3 nm compared to the original citrate-stabilized Au NPs, due to the refractive index changes in the vicinity of Au NPs.

Due to the protein coating, the Au@BSA NPs exhibit a pH-responsive reversible aggregation/disaggregation behavior. At pH values close to the pI of BSA (4.7<sup>44,45</sup>), Au@BSA NPs consistently aggregate and change their color from red over violet/purple to grey/blue. The plasmon band of the NPs' dispersion

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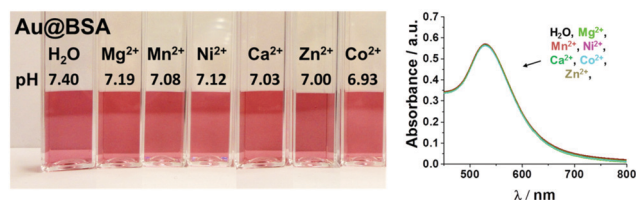
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becomes broader and shifts to longer wavelengths.<sup>37,40</sup> Aggregation can also be induced by the addition of metal ions because repulsive charges are screened.<sup>24,46</sup> In previous results, we investigated the interaction of Au@insulin and metal ions. Therein, we stated that overcharging was caused by the adsorption of ions.<sup>24</sup>

In this publication, we took a more precise look at the pH change by adding metal ions and at the influence of the metal hydroxide formation on the charge of the Au@BSA NPs. For this purpose, the interactions of Au@BSA NPs with different di- and trivalent metal ions were investigated. We selected the two alkaline earth metal ions  $Mg^{2+}$  and  $Ca^{2+}$  as divalent ions as well as the most commonly investigated transition metal ions, *i.e.*  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Zn^{2+}$  and  $Cu^{2+}$ , and the toxic heavy metal ions  $Pb^{2+}$ ,  $Cd^{2+}$  and  $Hg^{2+}$ . For the case of trivalent ions, we selected  $Al^{3+}$  as a main group metal ion and  $Fe^{3+}$ ,  $Cr^{3+}$ ,  $Au^{3+}$  and  $La^{3+}$  as transition metal ions.

To allow a comparison between this and the previous publication's data, the pH was adjusted to 7.4. Then the ions were added to a final concentration of 100  $\mu M$  and the pictures were taken after 30 min of incubation. Then the pH and the  $\zeta$ -potential were measured.



**Fig. 1** Optical response of Au@BSA NPs towards divalent metal cations. Photographs and respective UV-Vis spectra of dispersions of Au@BSA in the presence of various divalent cations confirmed the maintained stability indicated by the sustained red color. The cuvettes were sorted by decreasing pH from 7.4 to 6.9. The NP concentration for all dispersions was 0.15 mM Au(0) and the cation concentration was 100  $\mu M$ . The photographs and the UV-Vis spectra were taken 30 min after the addition of the cations.

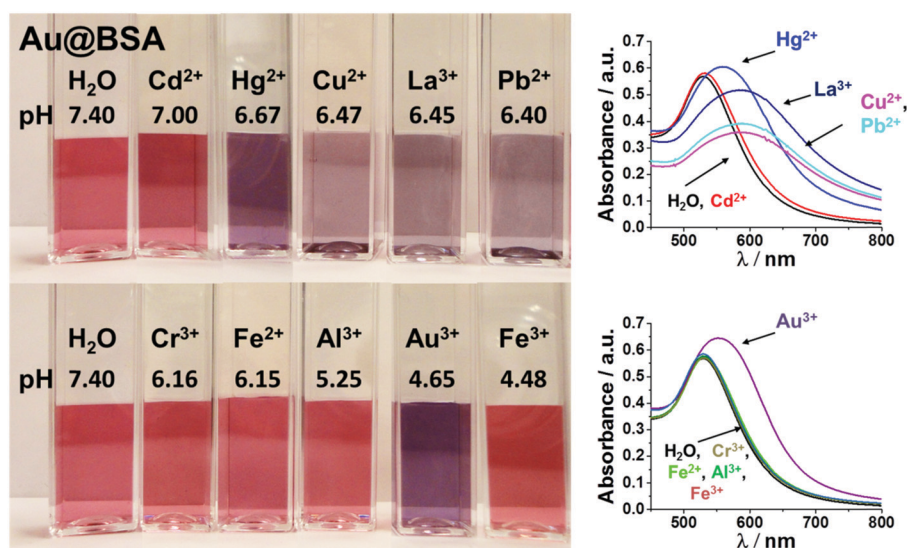
The cuvettes were organized with respect to their response and the resulting pH. In Fig. 1, Au@BSA dispersions are shown, which remained stable after the addition of the divalent metal ions ( $Ca^{2+}$ ,  $Mg^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Mn^{2+}$ ,  $Zn^{2+}$ ), *i.e.* they kept their original red color and the LSPR band was unchanged. The pH changed only very little from 7.4 down to 6.93.

In contrast to this, Fig. 2 shows the Au@BSA NP dispersions, that interact with some of the divalent ions ( $Cd^{2+}$ ,  $Hg^{2+}$ ,  $Cu^{2+}$ ,  $Pb^{2+}$ ,  $Fe^{2+}$ ) as well as with the trivalent ions ( $La^{3+}$ ,  $Cr^{3+}$ ,  $Al^{3+}$ ,  $Au^{3+}$ ,  $Fe^{3+}$ ). The optical response towards  $Fe^{2+}$  and  $Cd^{2+}$ ,  $Fe^{3+}$ ,  $Al^{3+}$  and  $Cr^{3+}$  was very weak and the LSPR band red-shifted only very slightly, whereas the particle systems showed a spontaneous color change from purple/blue to bluish grey in the presence of  $Hg^{2+}$ ,  $Cu^{2+}$ ,  $Pb^{2+}$ ,  $La^{3+}$  and  $Au^{3+}$  (Fig. 2), thus indicating strong aggregation of the NPs.

The pH changed from 7.4 to 7.00–5.25. The addition of  $Au^{3+}$  changed the pH to the isoelectrical point – for  $Fe^{3+}$ , the isoelectrical point was even exceeded. The strong change in pH stems from the ion specific Lewis acidity.

In order to detect overcharging and to get insight into the electrostatic stabilization of colloids, we conducted  $\zeta$ -potential measurements (Fig. 3). As stability threshold we applied  $\pm 25$  mV, which is common practice because it represents a Coulomb energy of  $1kT$ , *i.e.* the average kinetic energy of a charged particle. Therefore it solely applies for electrostatically stabilized NPs.<sup>47</sup> As steric repulsion contributes to the stabilization of Au@BSA NPs as well, it serves as an orientation here (*cf.* Fig. 3 red dotted lines).<sup>48,49</sup>

From Fig. 3A we can see that the  $\zeta$ -potential for Au@BSA NPs at pH 7.4 and at low ionic strength is beyond  $-40$  mV, indicating high surface charge and therefore high colloidal stability. In the presence of  $Mg^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$ ,  $Ca^{2+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$  and  $Cd^{2+}$ , the  $\zeta$ -potential of the NPs decreases to the stability threshold of  $-25$  mV. This confirms the colloidal stability of the Au@BSA NPs, which is indicated by the red color of the dispersions (*cf.* Fig. 1). For  $Cu^{2+}$ ,



**Fig. 2** Optical response of Au@BSA NPs towards di- and trivalent metal cations. Photographs and respective UV-Vis spectra of dispersions Au@BSA in the presence of various cations with decreasing pH from 6.7 to 4.5. The NP concentration for all dispersions is 0.15 mM Au(0) and the cation concentration is 100  $\mu M$ . The photographs and the UV-Vis spectra were taken 30 min after the addition of the cations.



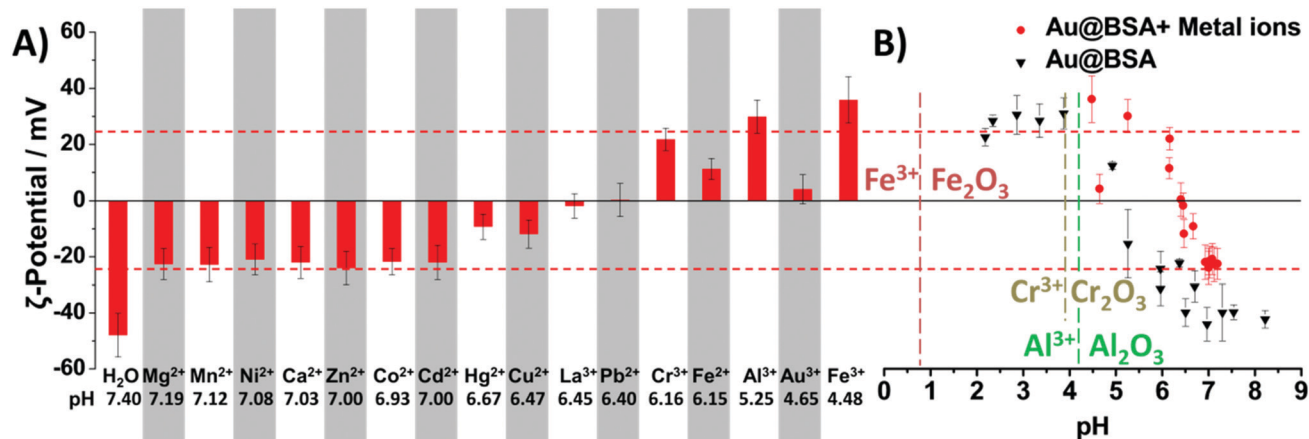


Fig. 3 (A)  $\zeta$ -Potential and pH values of Au@BSA measured immediately after mixing them with various metal ions. The dashed lines (red) at the  $\zeta$ -potential values of  $-25$  and  $+25$  mV indicate the stability threshold. (B) Comparison of the  $\zeta$ -potential values of Au@BSA with Au@BSA after the incubation with several ions. The thermodynamic stable species of the ions and their conversion point (pH) into their (hydr)oxides was plotted for the type of ions for which charge inversion occurred, e.g.: Cr<sup>3+</sup> (beige), Al<sup>3+</sup> (green) and Fe<sup>2+/3+</sup> (red).

Hg<sup>2+</sup>, La<sup>3+</sup> and Pb<sup>2+</sup> we could detect a reduction of the  $\zeta$ -potential up to around 0 mV which can explain the aggregation of the NPs.

A clear trend is visible when comparing the pH with the  $\zeta$ -potential of Mg<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, Ca<sup>2+</sup>, Zn<sup>2+</sup>, Co<sup>2+</sup>, Cd<sup>2+</sup>, Cu<sup>2+</sup>,

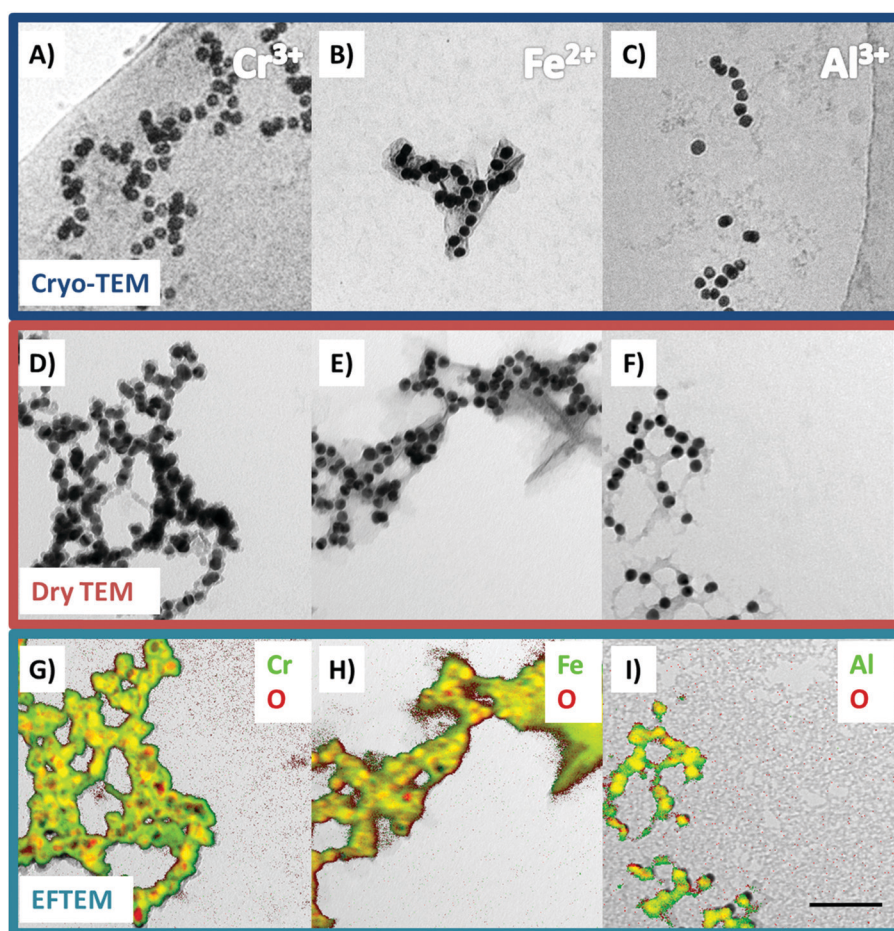


Fig. 4 Representative TEM micrographs of Au@BSA after the incubation with the metal ions that caused overcharging. (A–C) Cryo-TEM images of the Au@BSA NPs after the incubation with the metal ions. A grey shell can be seen around the Au@BSA NPs and in its surroundings. (D–F) Zero loss images of dried TEM samples which were used for the element mapping. (G–I) Element mapping (red = oxygen, green = metal ion, yellow = interference) by EFTEM which shows the presence of hydroxides in the grey shell. Scale bar is 100 nm.





$\text{Hg}^{2+}$ ,  $\text{La}^{3+}$  and  $\text{Pb}^{2+}$  (cf. Fig. 3). As expected, the  $\zeta$ -potential decreases with decreasing pH. A comparison of these  $\zeta$ -potentials with those of the pure Au@BSA NPs at the respective pH (cf. Fig. 3B), exhibits that the values after the interaction with ions are always lower than those of the pure Au@BSA NPs. That shows that the reduction in  $\zeta$ -potential is not only due to the pH change, but also caused by charge screening of the ions.

Furthermore, positive  $\zeta$ -potentials were measured for  $\text{Fe}^{2+}$ ,  $\text{Cr}^{3+}$ ,  $\text{Al}^{3+}$ ,  $\text{Au}^{3+}$  and  $\text{Fe}^{3+}$ , i.e. a charge inversion occurred. Here the  $\zeta$ -potential after the incubation with  $\text{Au}^{3+}$  is close to zero ( $4 \pm 5$  mV). For  $\text{Fe}^{2+}$  ( $11 \pm 4$  mV) and  $\text{Cr}^{3+}$  ( $22 \pm 4$  mV), the  $\zeta$ -potential is below or close to the stability threshold of 25 mV, whereas for  $\text{Al}^{3+}$  ( $30 \pm 6$  mV) and  $\text{Fe}^{3+}$  ( $36 \pm 8$  mV), the  $\zeta$ -potential values are above the stability threshold.

Overcharging of pure proteins is usually explained by the adsorption of ions at proteins.<sup>1,5,33,50–62</sup> However, this argumentation falls short, as the pre- and post-incubation pH has rarely been considered for Au@protein systems<sup>63–65</sup> or for pure proteins.<sup>9,20</sup> When the post incubation pH was measured, huge pH effects were observed that changed the protein charge drastically.<sup>12–14,22</sup>

As the pH is an important factor for the charge of proteins and thus also for Au@BSA NPs, the  $\zeta$ -potential values must always be assessed in the context of the environmental pH. For  $\text{Au}^{3+}$  and  $\text{Fe}^{3+}$ , the pH lies below the isoelectrical point so that a charge inversion can be due to the pH change. Positive  $\zeta$ -potential values at pH values above the isoelectrical point could be observed for  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$  (cf. Fig. 3). As the isoelectrical point of our Au@BSA systems is around pH = 5,<sup>40</sup> these positive charges cannot solely be due to the changes of pH.

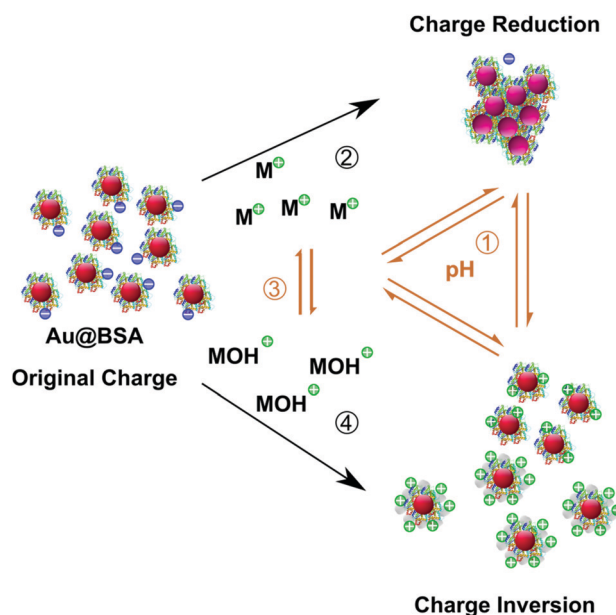
Therefore, this charge inversion must be due to the addition of cations or positively charged hydroxides.<sup>28,66–69</sup> Up to now, it is unknown whether adsorbed hydrolyzed ions<sup>25–33</sup> or adsorbed hydroxides<sup>34–36</sup> are responsible for overcharging. As the commonly used electrophoretic measurements *alone* cannot distinguish between hydrolyzed ions and hydroxides, the origin of charge inversion remains unclear.

The occurrence of hydroxides is ion specific and depends on the three parameters redox potential, ion concentration and pH. The boundaries of the thermodynamic stable species can be calculated for their respective parameters by using the Gibbs free energy and they can be visualized in Pourbaix diagrams.<sup>7,70,71</sup> The Pourbaix diagram shows the boundaries between metal ions and metal oxides, which occur as hydroxides in aqueous solution. Pourbaix diagrams are commonly used in engineering science to predict corrosion. To the best of our knowledge this is the first time that they are applied in the discussion of overcharging in colloidal science.

Fig. 3B shows the boundary pH values for  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$  at the applied ion concentration of 100  $\mu\text{M}$  and at the redox potentials of oxygen-saturated water. Oxygen saturation can be assumed as the experiments were conducted under ambient conditions. Fig. 3B shows that hydroxides can be expected for all of the three ions. As the hydroxides are positively charged at the given pH values<sup>8</sup> and the NPs are negatively charged, an interaction between the two occurring species can be expected.

To detect if adsorbed ions or adsorbed hydroxides are present and cause the overcharging, cryo-TEM and EFTEM was applied (cf. Fig. 4).

The cryo-TEM micrographs of  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$  (Fig. 4A–C) show a grey shell around the Au@BSA NPs. This shell also connects the NPs with each other and is found in their further surroundings. In comparison to the cryo-TEM images of the pure Au@BSA NPs (cf. Fig. S1, ESI<sup>†</sup>), a significant increase of aggregates occurred. This can be due to the insufficient surface charge or it was caused by the charge inversion process. Charge inversion of colloids can generate aggregates, because NPs reach and cross the isoelectrical point, also known as the “point of zero charge”. Here the charge repulsion is at a minimum and thus leads to aggregation.<sup>72</sup> To prove that the grey shell consists of hydroxides, we conducted element mapping of each respective ion and oxygen by energy filtered TEM. Au could not be mapped due to high energy loss edges ( $> 2000$  eV).<sup>73</sup> To visualize the results of the element mapping we overlayed the zero loss images (Fig. 4D–F) with the element maps (Fig. 4G–I). In the zero loss images the same grey shells could be observed as in the cryo-TEM measurements. The element mapping shows that the regions of



**Scheme 1** Describing the effect of ions with Au@BSA NPS requires to take into account 4 different equilibriums, which are interconnected. Therein, the pH is the key parameter as it is involved in equilibrium 1 and 3 (brown color) directly. (1) The charge of Au@BSA NPs is influenced by the pH: above the isoelectrical point, the NPs are negatively charged and stable in dispersion, which is indicated by their red color. A reduction of the pH leads to aggregation around the isoelectrical point. The solution turns violet. Further reduction of the pH then leads to charge inversion, generating stable Au@BSA NPs again. (2) The charge of Au@BSA NPs can be screened by the addition of metal ions. Depending on the sort of ion and on the concentration, this can lead to aggregation. (3) The addition of metal ions can influence the pH. Depending on the pH, metal ions can convert into hydroxides. These hydroxides are positively charged at or below neutral pH. (4) For  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Al}^{3+}$  overcharging is induced by a shell of adsorbed hydroxides on the Au@BSA NPs.



the grey shell consist of oxygen and the respective metal ion. This proves that the outer shell of the colloids consists of hydroxides. As the outer shell determines the charge of a colloid these measurements prove that overcharging is due to hydroxides on the Au@BSA NPs.

Usually hydroxides are known to occur in the form of a white precipitate. As the hydroxide objects are below visible wavelength in this study, they could not be seen in the NPs' dispersion with the bare eye (*cf.* Fig. 2). These findings support the theory of hydroxides being responsible for the overcharging rather than hydrolyzed ions.<sup>34–36</sup>

### 3. Conclusion

We investigated the influence of metal ions being added to Au@BSA solution. Focus was put on the resulting pH and the charge of Au@BSA. The results are summarized in Scheme 1.

The surface charge of the Au@BSA NPs strongly depends on the pH (*cf.* Scheme 1.1 and Fig. 3B). The majority of the metal ions (*i.e.*  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cd}^{2+}$ ,  $\text{Hg}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{La}^{3+}$  and  $\text{Pb}^{2+}$ ) show a pH reduction (*cf.* Scheme 1.3 and Fig. 3) and charge screening (*cf.* Scheme 1.2 and Fig. 3) which leads to a reduction in surface charge. A charge inversion from negative  $\zeta$ -potential values to positive  $\zeta$ -potential values was observed for  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Al}^{3+}$ ,  $\text{Au}^{3+}$  and  $\text{Fe}^{3+}$ . For these ions, the pH changed drastically from 7.4 to 6.16–4.48. For  $\text{Cr}^{3+}$ ,  $\text{Fe}^{2+}$  and  $\text{Al}^{3+}$ , the pH values were well above of the isoelectrical point of Au@BSA, so that the pH cannot be the cause of the charge inversion. Pourbaix diagrams were used to show that at the respective pH of these ions, hydroxide formation can be expected (Fig. 3B and Scheme 1.3). For these systems, a shell of adsorbed hydroxides was found on the Au@BSA NPs (Scheme 1.4 and Fig. 4). As the hydroxides are positively charged below neutral pH, they render the Au@BSA NPs positive.

These results show that hydroxides – not unhydrolyzed or hydrolyzed ions – are responsible for the phenomenon of charge inversion. We showed that electrophoretical measurements alone ( $\zeta$ -potential) cannot distinguish between the different species. To do so, it is necessary to combine those measurements with a microscopic technique such as TEM. Our findings provide fundamentally new insights into protein and metal ion interactions as well as into their correlations with the pH.

### 4. Experimental section

#### Chemicals

$\text{HAuCl}_4 \cdot 3\text{H}_2\text{O}$  ( $\geq 99.9\%$ ), tri-sodium citrate dihydrate ( $> 99\%$ ), bovine serum albumin (BSA), and all the metal salts ( $\geq 99.0\%$ ) were purchased from Sigma-Aldrich. The solutions of metal ions were prepared from  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{ZnCl}_2$ ,  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{Ni}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{CoNO}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{Pb}(\text{NO}_3)_2$ ,  $\text{Cd}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{HgCl}_2$ ,  $\text{La}(\text{NO}_3)_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ ,  $\text{CrCl}_3$ ,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  by separately dissolving each compound in water. All chemicals were used as received. Milli-Q grade water was used in all preparations.

#### Synthesis of Au@protein NPs

Citrate coated Au NPs (Au@citrate) with an average particle size of 15 nm were synthesized by the Turkevich method.<sup>74</sup> The NPs were then coated with proteins by a simple ligand exchange process. For this, a protein–citrate solution was prepared with a concentration of  $1 \text{ mg mL}^{-1}$  protein and  $0.1\%_{\text{wt}}$  citrate. The pH of the solution was then adjusted to 7–8 with NaOH. Subsequently, a 10-fold volume of citrate coated Au NPs ( $[\text{Au}] = 0.5 \text{ mM}$ ) were added under vigorous stirring. Resulting in a final protein concentration of  $0.1 \text{ mg mL}^{-1}$ . The mixture was stirred for 24 hours at room temperature. Finally, the protein coated Au NPs (Au@protein) were purified *via* 5-fold centrifugation ( $10\,000g$ , 30 min) and stored at  $\text{pH} \geq 9$  at  $4^\circ\text{C}$ .

#### Characterization

TEM images were acquired on a Libra 120 cryo-TEM from Carl Zeiss NTS GmbH equipped with a LaB6 source and an omega-type energy filter. The acceleration voltage was 120 kV. For TEM measurements, the samples were prepared by drop-coating  $5 \mu\text{L}$  of the NPs' solution onto a carbon coated copper grid (CF200-Cu, Electron Microscopy Sciences, Hatfield, USA). After 10 min, residual solution was removed by blotting with a filter paper. Presence of iron, chrome, aluminum and oxygen and their spatial distribution were determined by electron energy loss spectroscopy (EELS) and energy filtered TEM imaging (EFTEM). UV-Vis-near-IR spectra were measured with a Cary 5000 UV-Vis-NIR spectrophotometer.  $\zeta$ -Potential values were determined through electrophoretic mobility measurements using a Malvern Zetasizer Nano ZS by taking the average of five measurements, each consisting of 70 runs. pH measurements were performed with SI Analytics Lab 850 equipped with a N6280. The photographs were recorded with a Sony DSC-W350 digital camera.

#### Metal ion study of Au@protein NPs

Prior to the mixing of the metal ions with the Au@protein NPs (Au concentration =  $0.15 \text{ mM}$ ), the pH of the NP dispersions were adjusted to  $\text{pH } 7.4 \pm 0.1$ . Then  $2 \text{ mL}$  of the NP dispersion were given in a cuvette (polystyrene,  $10 \text{ mm}$ ) and  $20 \mu\text{L}$  of the dispersion was replaced by  $20 \mu\text{L}$  of a freshly prepared  $10 \text{ mM}$  solution of each metal ion, resulting into the final metal ion concentration of  $100 \mu\text{M}$ . The solutions were mixed directly in the cuvettes by pipetting the mixture up and down multiple times. Then UV-Vis spectra, photographs and the  $\zeta$ -potential measurements were conducted.

### Conflicts of interest

There are no conflicts to declare.

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