A NOVEL PROCESS FOR DETECTING HUMAN HAEMOGLOBIN VARIANTS

FIELD OF INVENTION

The present invention relates to a process for detecting the presence or absence of human hemoglobin HbA0 and/or HbE in a sample by way of nanoparticle - hemoglobin interaction.

BACKGROUND OF INVENTION

Metal ions accumulate in the brain with aging and in several neuro degenerative diseases (Zhengrong C et al, Eur J Pharm Biopharm 2005, 59, 263-272). Aside from Wilson's disease (Kim JM et al, Neurosci Lett 2005, 382, 143-147), which results from copper storage, recent attention has focused on the accumulation of zinc, copper and iron in the Alzheimer's disease (AD) brain (Sadrzadeh SM et al, Am J Clin Pathol 2004, 121, S64-S70) and the accumulation of iron in parkinson's disease. In particular, the parenchymal deposition of beta-amyloid (Abeta) and its interaction with metal ions has been postulated to play a role in the progression of AD (Shi Z et al, World J Gastroenterol 2005, 11, 3691-3695). It is thus imperative, that there is a coupling between the aggregation of proteins and metal particle, which can form in various physiological conditions. The question of the exact angstrom or nanometer scale in which the metal clusters occur in the physiological milieu, remains unanswered. However, an in vitro study of metal nanoparticle interaction can be an important step towards conceptualization of the actual physiological process.

Literature reports on nanoparticle macromolecular interactions have emphasized optics based nano-sensors or disease (Popovtzer R et al, Nano Lett 2005, 5, 1023-1027) or the selective adsorption of proteins on a nanoparticle surface (Soonwoo et al, Chem Biol 2005, 12, 323-328). Recently, Zare and his group have shown how different unfolding states of a heme protein (cytochrome C) differentially interact with gold nanoparticles ((Soonwoo et al, Chem Biol 2005, 12, 323-328). The importance of nanoparticle in Zare's work lies primarily in sensing of folding states. One however needs to have a deeper understanding of the interplay between the protein and the nanoparticle, particularly, how the nano assembly is affected by the protein and how the protein reciprocates it.

OBJECTS OF THE INVENTION

The primary object of the present invention is to disclose a process for detecting the presence or absence of human hemoglobin HbA0 and/or HbE in a sample by the way of nanoparticle-hemoglobin interaction.

Another object of the present invention is to disclose a process in which the nanoparticle protein cross talk helps to separate out specific proteins even if they have very close homology (like the discrimination of HbA0 from HbC or HbE from HbA2).

Still another object of the present invention is to develop a process which has diagnostic ability to differentiate among proteins by the extent of their unfolding.

SUMMARY OF THE INVENTION

The present invention is based on the unexpected finding that clusters of copper nano particle (average cluster size 164nm) undergo a multi step transition when allowed to interact with HbA0, the normal fraction of human hemoglobin. In the first stage of this interaction the nano particle clusters are broken and dynamic light scattering studies indicate that the particles reach a smaller size (~ 4nm). This follow a capture of nanoparticles by hemoglobin. The capture populates the molten globule state and a resultant aggregation of protein occurs. As the hydrodynamic size of the aggregates exceeds 1.4 μm the complex precipitates out along with the nanoparticles.
A reversal of zeta potential of the protein-nanoparticle complex from - 11 mV to + 13 mV is also observed in the present invention. The aggregation is highly specific towards A0 fraction of human hemoglobin (HbA0), while fractions like HbC show no response. Similarly, other proteins e.g. serum albumin or haptoglobin do not exhibit such large alteration in water solubility on exposure to equivalent (or higher) concentration of nanoparticles. These specific nanoparticle induced protein solubility changes have potential use in proteomic problems.

The present invention shows that protein has a surfactant property that declusters a loosely formed (say by van der Waals interaction) nanoassembly. On the other hand, the isolated nanoparticles populate the molten globule state of the protein (in a protein specific way), that triggers protein aggregation. The energy landscape by its very nature is dependent on the surface geometry of the contact boundary and thus the possibility of inducing aggregation of specific proteins by a specific set of nanoparticle is not ruled out.

The induced unfolding measured from the peroxidase activity of the heme, mimics, the unfolding induced by denaturing agents (e.g. guanidium hydrochloride).

Unlike the denaturant however, the unfolding activity induced by copper nanoparticles has protein specificity.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1: Size distribution of nanoparticles, nanoparticle-hemoglobin complex and their respective size changes.

Figure 2: The right panels show spectrum of copper nanoparticle solution at two different intervals of time separated by 10 minutes. Each spectral set contained 240 cycles of individual spectrum taken after a time delay of 5 seconds. The left panel represents the temporal dynamics of absorbance at 200nm.

Figure 3: Comparison of soluble fraction of hemolysate before (upper panel) and after (lower panel) addition of copper nanoparticles. The soluble fraction is prepared after pre-incubation with 1.5 ppm of copper nanoparticles for 1 hour and then centrifuging at 4000g to remove aggregates. High-pressure liquid chromatography is run using the Chrom system it under identical flow conditions.

Figure 4: Peroxidase activity of hemoglobin, nanoparticle and hemoglobin and nanoparticle mixture. **I:** Activity of nanoparticle (1.5 ppm) only; **II:** Activity of hemoglobin (0.4 mg/ml); **III:** Activity of hemoglobin (0.4 mg/ml) + nanoparticle (0.5 ppm); **IV:** Activity of hemoglobin (0.4 mg/ml) + nanoparticle (1.5 ppm).

Figure 5: Gel electrophoresis pattern of hemoglobin (Hb)-copper nanoparticle (CuNP) interaction. A: The gel electrophoresis pattern of Hb and of Hb in the presence of different concentrations of CuNPs. Lanes are as follows: **A,** Hb (0.4 mg/mL); B, Hb (0.4 mg/mL) + CuNPs (1.8 ppm); C, Hb (0.4 mg/mL) + CuNPs (0.3 ppm); D, Hb (0.4 mg/mL) + CuNPs (0.6 ppm); E, Hb (0.4 mg/mL) + CuNPs (0.9 ppm); F, Hb (0.4 mg/mL) + CuNPs (1.2 ppm); G, Hb (0.4 mg/mL) + CuNPs (1.5 ppm). **B,** Analysis of the gel is performed using a digital image. The image is converted into an image matrix using MATLAB 7.1. The average pixel strength (I) is measured using the IMPIXEL command, and the intensity is expressed by *225-I* and is plotted against different CuNP concentrations.

Figure 6: Gel electrophoretic analysis of serum-copper nanoparticle (CuNP) interaction. **A,** the gel electrophoresis pattern of serum in the presence of

different concentrations of CuNPs. Lanes are as follows: A, serum (0.4mg/l); B, serum (0.4 mg/ML) + CuNPs (0.6 ppm); C; serum (0.4 mg/mL)n + CuNPs (1.2 ppm); D, serum (0.4 mg/mL) + CuNPs (1.8 ppm). **B,** The band marked 1 corresponds to the bottom-most gel band and represents human serum albumin. The other bands, respectively, (from bottom to top) represent α-globulin (band 2), β-globulin (band 3) and γ-globulin (band 4). It can be seen that the integrated intensity of the bands remains unchanged with increasing concentration of CuNPs. Intensity is evaluated using analysis of the digitized image as described for Figure 5.

Figure 7: Zeta potential-dependent phase of copper nanoparticles (CuNP), CuNPs plus hemoglobin and their changes with time, represented in different colors.

Figure 8: SEM image created with samples containing copper nanoparticles (CuNPs) and CuNPs + HbA0.

DETAILED DESCRIPTION

In the present invention clusters of the Cu nanoparticles have preferably a size of 160 nm. The clusters are immediately broken when the protein (HbA0) is added to the nanoparticle suspension. Figure 1 shows initial de-clustering of the nanoparticles at different intervals of time. This is followed by formation of extended structure of the protein [see different colored dynamic light scattering (DLS) size graphs in Figure 1]. Longer incubation with Cu nanoparticles leads to aggregation with the formation of micron sized structure, which immediately precipitates out of the solution.

The right panels of Figure 2 show spectrum of the copper nanoparticle solution which shows an optical absorbance around 220nm at two different intervals of time. The left panels show the temporal dynamics of the peak absorbances. While the upper left panel shows an increase of peak intensity with time, the lower left panel shows a decrease. The sporadic decrease in absorbance in the first phase (upper left panel) is reflected by the rather uniform decrease of peak intensity in the right panel. This is a reflection of the events described in Figure1. While the increase in peak intensity in response to addition of protein implies more efficient optical absorbance, as a result of de-clustering of the nanoparticle the decrease represents protein aggregation.

The net effect of cross talk between the copper nanoparticle and the hemoglobin is best understood by the following experiments that successively confirm the respective solubility decrease of nanoparticles and the protein as a result of the interaction. The comparative HPLC profiles of human hemoglobin (hemolysate) in presence and absence of nanoparticles is respectively shown in upper panel and lower panel of Figure 3. Interestingly in the lower panel the HbA0 peak disappears and the peaks remaining in the soluble fraction correspond to HbC and HbF [peak 20, identified by Chrome Systems (personal communication]). This clearly shows that the nanoparticle protein interaction that results in the aggregation and precipitation is specific to HbA0. The major hemoglobin component.

The fact that the extended structure formation that is followed by aggregation is mainly due to misfolding is also demonstrated by the enhancement of peroxidase activity of the heme moiety in presence of nanoparticles. Figure 4 shows that at a particle concentration of 0.5 ppm the activity is enhanced and at higher nanoparticle concentration (1.5 ppm) the activity falls below the control. This apparently paradoxical result is resolved by considering that at increased concentration of nano particles the nanoparticle protein complex undergoes precipitation. As a result, the effective protein concentration (responsible for the peroxidase activity) reduces to the extent that is not compensated by the unfolding induced increase in peroxidase activity. The increase in activity is also observed if the protein solution is subjected to unfolding by a denaturant like guanidium chloride.

The fact that the presence of nanoparticle populates a particular folding state of the protein is further evident from the native electrophoresis pattern obtained in presence and absence of nanoparticles (Figure 5). In Figure 5 as one goes from lanes A, B, C, D with stepwise increasing nanoparticle concentration there is an increase in pixel intensity of one band (marked by arrow in the Figure 5). Interestingly, the protein specific nature of the interaction is revealed from comparison of serum proteins (Figure 6) in presence and absence of nanoparticles that show no significant alteration in either band position or intensity ion presence of copper nanoparticle.

The protein nanoparticle interaction also leads to temporally varying zeta potential changes. The Laser Doppler velocimetry measurement, presented in Figure 7, shows the velocimetric phase plots at different time intervals. Figure 7 shows how the initial declustering and subsequent clustering of the nano particles are associated with the alterations in the zeta potential. Interestingly the corresponding Zeta

potential undergoes an alteration from -22 mv to+14mv. The zeta potential dynamics is sensitive to the dilution of the nanoparticles. It is also observed that at higher dilution there is an initial shift towards higher negative zeta potential (associated with nanoparticle declustering).

Apart from this utilitarian aspect, the point that needs deeper theoretical insights, is the protein induced alteration of zeta potential that has been reported for the first time (in the present invention) in the context of protein-nanoparticle interaction. The zeta potential change is explained by (a) conformational rearrangement of the nanoparticle-protein complex that leads to partial shielding of charge; (b) an altered band structure of the nanoparticles that populates or depopulates their valance or conduction band in presence of the coupling bimolecule. The failure of some heme proteins to respond to the Cu nanoparticles that include some Hb variants (HbC, HbF etc) may be because the Cu nanoparticle-Hb interaction occurs only when the alpha and beta chains are properly assembled (like in the HbA0 fraction). The emergence of protein aggregation caused by misfolding of protein indicates that the nanoparticles drive the protein in a molten globule state that facilitates the aggregation.

Figure 8 depicts the SEM (Scanning Electron Microscopy) images of the copper nanoparticles in the presence and absence of HbA0. It is observed that, whereas the copper nanoparticles remain as dispersed clusters with a certain distribution in size, the presence of proteins leads to self-assembly. This figure provides direct evidence of the cross-talk between the copper nanoparticles and hemoglobin. Importantly, the dispersed copper nanoparticle clusters (in the absence of proteins) corresponds to the DLS measured values of approximately 160 nm. The self assembled structure, being the final state, shows a mosaic of copper nanaoparticles and the protein.

The process of differentiating normal hemoglobin from hemoglobin variants is described hereinafter with examples, which are illustrative and are not intended to be taken restrictively to imply any limitation on the scope of the present invention.

EXAMPLE 1:

Aggregation of Cu nanoparticle in presence and absence of protein

The confirmation that the aggregation process includes the nanoparticles along with the protein comes from atomic absorption spectrometry (AAS). The AAS measurement

described in Table 1 below clearly indicates that around 50% of the copper disappears from the nanoparticle suspension in presence of protein:

Table 1: Atomic absorption analysis of Cu Nanoparticle in presence and absence of protein to find the percentage of nanoparticle protein assembly that is precipitated out of the solution (marked by A, B and C)

Cu nanoparticle (Np) dilution (ppm)	CuNp (ppm)	CuNp +HbA0	%CuNp bound to HbA0	CuNp+ hemolysate	%CuNP bound to hemolysate
A.	4.5	2.7	40.0	1.8	60
B.	1.5	0.9	40.0	0.8	46
C.	0.5	0.5	0.0	0.5	0.0

The effect however is totally absent if the nanoparticle solution is diluted beyond a certain level (last row of the Table).

EXAMPLE 2:

Detecting different classes of hemoglobinopathy

The use of the present invention in detecting different classes of hemoglobinopathy is studied. HbE is collected from hemolysate of patients homozygous for HbE and this hemolysate also contains HbA0. Cu nanoparticles with 4.5 ppm copper are added to 0.2 ml of hemoglobin (concentration 0.4 mg/ml). Results of high-pressure liquid chromatography of Cu nanoparticles added to different variants of hemoglobin are shown in Table 2.

Table 2: Results of high-pressure liquid chromatography of Cu nanoparticles added to different variants of hemoglobin

Variants	Cu nanoparticles volume (μl)	R
HbA0	200	13
HbA2	200	37.5
HbE	200	5
HbA0	400	1.3
HbA2	400	2.5
HbE	400	0.0

"R" represents the ratio of the area of the HPLC peaks of the remaining fraction after centrifuging the Cu nanoparticles-treated protein are considered.

The observation which can be seen with the naked eye is summarized as follows. Very little or practically no precipitation occurs in case of HbA2, moderate precipitation in case of HbA0 and extensive precipitation in case of HbE.

EXAMPLE 3:

Direct evidence of cross-talk between copper nanoparticles and hemoglobin

Figure 8 shows the SEM (Scanning Electron Microscopy) images of the copper nanoparticles in the presence and absence of HbA0. It is observed that, whereas the copper nanoparticles remain as dispersed clusters with a certain distribution in size, the presence of proteins leads to self-assembly. This figure provides direct evidence of the cross-talk between the copper nanoparticles and hemoglobin. Importantly, the dispersed copper nanoparticle clusters (in the absence of proteins) corresponds to the DLS measured values of approximately 160 nm. The self assembled structure, being the final state, shows a mosaic of copper nanaoparticles and the protein.

The main advantages of the present invention are:

1. Conventional nano device application suffers from defects that form even in most resilient nano structure such as carbon nano tubes, whereas the proteomic application of the present invention is free from this defect sensitivity.
2. The extent of sensitivity that is necessary for the cross interaction between the nanoparticle and the protein is specific to the protein.
3. An important but simple advantage of the present invention is that the nanoparticle protein cross talk helps to separate out specific proteins even if they have very close homology. This serves as additional dimension to proteomic characterization of protein mixtures.
4. The present invention has important diagnostic implications of the ability to differentiate among proteins by the extent of their unfolding. The specific nanoparticle-induced protein solubility changes have use in proteomic problems.
5. The present invention has important applications in hemoglobinopathy in particular and proteomic applications in general.