

## Peptides as capping ligands for *in situ* synthesis of water soluble Co nanoparticles for bioapplications

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**Abstract:** For the first time peptides were used as capping ligands for *in situ* synthesis of water-soluble cobalt nanoparticles (NP) with the aim to use these particles for biological applications. In this paper, we describe the synthetic method, and characterization of the samples by transmission electron microscopy (TEM), X-ray powder diffraction (XRD) and superconducting quantum interference device (SQUID) magnetometry. The peptides were found to facilitate the formation of nanoparticles and partly protect the nanoparticles from oxidation.

### 1. Introduction

In small magnetic crystals, all the atomic spins are coupled together to create a giant magnetic moment, and behave as a single magnetic domain. The magnetic interactions between the particles and external field are long range, hardly screened and do not affect biological tissue. Magnetic nanoparticles have many potential applications in biomolecular sciences, e.g. contrast enhanced agents for magnetic resonance imaging (MRI), targeted therapeutic drug delivery and hyperthermia treatment for cancers, as well as building devices using a “bottom up” approach. Magnetic immunoassay techniques have been developed in which the magnetic field generated by the magnetically labeled targets is detected directly with a sensitive magnetometer [1], and more recently molecular interactions between biological molecules such DNA and proteins, as well as enzymatic reactions, were detected by spin-spin relaxation times using nuclear magnetic resonance (NMR)/MRI techniques [2].

For biomolecular applications, the magnetic NP should have the following characteristics: (i) large saturation magnetization and high magnetic susceptibility to respond sensitively to a small external magnetic field or the signal of a magnetic sensor; (ii) superparamagnetism at room temperature, i.e. the magnetic moment fluctuates freely in the absence of a magnetic field thereby cancelling the magnetic attraction between particles, to avoid aggregation; (iii) size comparable to bio-molecules. To overcome the relatively low saturation magnetization of ferrimagnetic iron oxides (magnetite Fe<sub>3</sub>O<sub>4</sub>

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and maghemite  $\gamma\text{-Fe}_2\text{O}_3$ ), large particles of few hundred nanometers to a few microns have been used. The relatively high mass of the large particles reduces substantially the efficiency of magnetic migration procedures. Moreover, because of their large size, the stability of the dispersed solution is compromised by the correspondingly increased Van der Waals interactions and the particles are subject to opsonisation by the immune system and so cleared rapidly when they are used *in vivo*. These materials are at the upper size of nanomaterials and considerably larger, (by a factor of 10-100), than most biomolecules [3] and so are not ideal for biomolecular sciences and “bottom up” assembly of devices. In contrast, transition metal, e.g. Co, have a much higher value of saturation magnetization, which allows much smaller NP while still retaining the same magnetic response/or sensitivity as those of the larger ferromagnetic iron oxides. There are several methods to synthesize cobalt NP in an organic environment with high monodispersity [4, 5]. However, these particles cannot be used in aqueous and biological environments due to their insolubility. When synthesis is performed in an aqueous medium [6, 7], poor cobalt NP are obtained, i.e. the NP are rather polydisperse with formation of complexes and antiferromagnetic CoO phase. The full exploitation of the remarkable properties of these transition metal magnetic NP requires complete stability in biological environments, including monodispersity and chemical stability towards oxidation. No current methods provide a means of synthesizing Co magnetic NP with these properties.

A capping ligand shell is required to stabilize the transition metal NP from aggregation and to make them water-soluble and stable in complex biological solutions containing multiple electrolytes and macromolecules. The recently discovered peptide capping ligands [8] provide a unique route to stabilization and functionalization of gold NP. The ability to tune the properties of the peptides (by varying the length, and sequence of amino acids) makes them a unique class of ligands for combinatorial nanomaterial synthesis. In addition to the 20 amino acids that occur naturally in proteins, over 100 unnatural amino acids are available for peptide synthesis, which provide access to a huge chemical combinatorial space. The approach described here is that peptides are used as ligands to control the nucleation and growth of cobalt NP during synthesis. Peptides with rationally chosen structural and functional groups are used as frameworks for controlled synthesis of NP and at the same time to provide the capacity to stabilize these NP in biological environments. The peptides play three important roles: (i) control of the nucleation and growth processes to produce the designed morphology (shape, size) and internal structure (crystallinity, crystal phases and composition) of NP; (ii) protect the NP/clusters from chemical degradation (oxidation, hydrolysis); and maintain their physical stability (dispersion) in aqueous and biological environments; (iii) allow biofunctionalization.

## 2. Materials and methodology

**2.1. Materials.** The peptide TLVNN (threonine-leucine-valine-asparagine-asparagine) was purchased from Pepsyn Ltd., Liverpool, the UK. The peptide was dissolved in toluene: DMF (dimethylformamide) (20:1, v/v). Di-cobalt octa-carbonyl  $\text{Co}_2(\text{CO})_8$  containing 1-5% hexane as a stabilizer and anhydrous DMF were purchased from Sigma-Aldrich Ltd., the UK. Anhydrous toluene was distilled in-house prior to use.

**2.2. Synthesis of Co NP in the presence of peptides as capping ligands.** The principle of the synthesis is to thermally decompose  $\text{Co}_2(\text{CO})_8$  in the presence of a designed peptide in a non-polar solvent such as toluene. All the syntheses have been carried out using standard airless techniques. Peptides dissolve poorly in non-polar solvents and may form extended structure in such solvents. The preferred solvent for peptide is polar one such as DMF. However, these solvents promote the formation of cobalt complexes during the reaction. A typical synthesis consisted of a penta-peptide (20 mg, 40  $\mu\text{mol}$ ) dissolved in 0.5 ml DMF, degassed with nitrogen and then diluted with 10 ml of toluene. Five ml of this solution was transferred to a reaction flask which had been degassed beforehand. The peptide was heated to the boiling temperature of toluene (110 °C) and refluxed under a flow of  $\text{N}_2$ . Cobalt carbonyl precursor solution (1ml), which was at room temperature, was injected rapidly into the middle of the

vigorously mixing solution. This produced a rapid heating of the cobalt carbonyl, which was decomposed to release cobalt atoms into the solution. Cobalt atoms are unstable in toluene so they nucleate and form clusters which grow and finally form nanoparticles. Samples were collected for analysis after all the particles were precipitated onto the reaction walls (between 15 min and 90 min).

*2.3. Experimental design to establish the role of peptides in the synthesis of Co NP.* As the role of the peptide in the medium during the synthesis of cobalt NP is unknown, control experiments were carried out to establish the interactions of the different components in the reaction:

Reaction 1:  $\text{Co}_2(\text{CO})_8$  + toluene + DMF + TLVVN.

Reaction 2:  $\text{Co}_2(\text{CO})_8$  + toluene + DMF determines the effect of DMF alone on Co NP growth.

Reaction 3:  $\text{Co}_2(\text{CO})_8$  + toluene + peptide determines the effect of peptide alone.

Reaction 4:  $\text{Co}_2(\text{CO})_8$  + toluene provides a baseline to identify how DMF and peptide in the reaction mixture modify the decomposition of  $\text{Co}_2(\text{CO})_8$  and the growth of Co NP.

*2.4. TEM.* A few drops of the sample from the above syntheses after dispersing in water were added to a carbon-coated TEM grid at room temperature, covered with a watch glass, and allowed to evaporate slowly. Images of the Co NP were obtained by bright-field and dark-field in a high resolution TEM (JEOL FX2000, Japan) operated at an accelerating voltage of 200 kV. Dark-field produces images by the diffracted electrons instead of the transmitted ones and provides direct observation of the metallic NP within the organic mater.

*2.5. XRD.* Powder samples were dispersed in hexane and then deposited on a quartz plate. Hexane was chosen, as it has low boiling point, so quick deposition of the sample can be obtained. XRD data was collected with a PANanalytical diffractometer using a wave length of 1.798 Å from Co  $K_\alpha$  radiation. The step size used was  $0.0167^\circ$  in  $2\theta$ , and the accumulation time for each sample was at least 2 h.

*2.6. Superconducting quantum interference device (SQUID) magnetometry* was employed to measure the temperature dependence of the zero field cooled (ZFC) and field cooled (FC) magnetization as well as the hysteresis loop. The measured sample was made of powder sealed in a gel cap.

### 3. Results and discussion

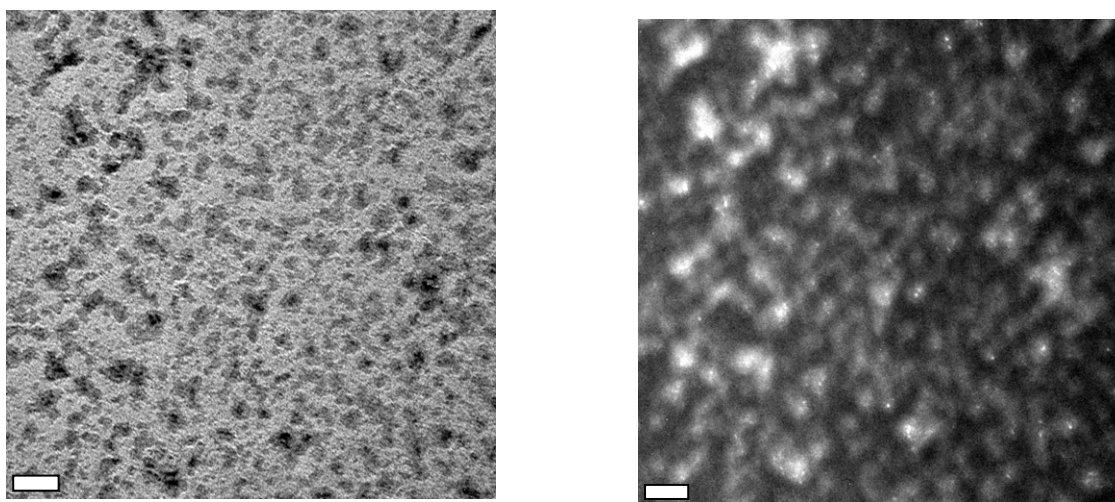
*3.1. Synthesis of Co NP in the presence of peptides as the capping ligands.* A few minutes after the reaction, a large amount of black powder deposited onto the reaction flask walls. During nucleation and growth of the NP, the functional groups (e.g., carboxyl, amine and thiol) of the peptides at the refluxing temperature of toluene may attach to the crystal surface of Co metal to form coordinate bonds. In a hydrophobic solvent, interpeptide bonding will be driven by the H-bonding potential of the amides of the five peptide bonds and the two asparagine side chains. By analogy with gold NP [8], it seems likely that, as the peptide form a monolayer on the Co nanocrystals, one of the polar termini ( $\text{NH}_2$  or  $\text{COOH}$ ) will be exposed to the solvent. This would render the NP insoluble in the non-polar solvent. Thus, after few minutes the particles precipitate out of the toluene onto the reactor walls where they form a black deposit. When the reaction was complete, following removal of toluene and addition of water, the cobalt suspension was easily recovered from the walls by a quick sonication (1 min) to yield a black coloured suspension containing colloidal Co NP.

*3.2. Visual observations.* Visual inspection of the reaction products and a simple examination of their response to an external magnetic field (by approaching a  $\text{Nd}_2\text{Fe}_{14}\text{B}$  magnet to the vial) provides a quick insight into their nature. Precipitated metallic cobalt nanometric powders are black.

Pink and blue coloured solutions indicate the presence of cobalt complexes. Solutions containing cobalt NP should appear between brown-grey to black. Precipitation and flocculation are taken as a sign of particle instability in the solvent. It is important to differentiate between reversible and irreversible aggregation. The former can be easily modified and it may not affect further experiments, while the latter renders the particles useless. Response of the colloid to the magnet is a test for the presence of metallic cobalt, which will be nanometric in solution and micrometric in the precipitates. Thus, a successful synthesis corresponds with a clear black solution which responds to a magnet and remains so during an extended period of time. In reactions 1, 3 and 4, black magnetic deposits were obtained. In reaction 2, a blue clear supernatant was obtained together with a black magnetic precipitate.

**3.3. Characterizations.** All these experiments were performed at least 4 weeks after the synthesis. Samples were dispersed in water, allowing water and oxygen to interact with the products of the synthesis and thus explore their stability.

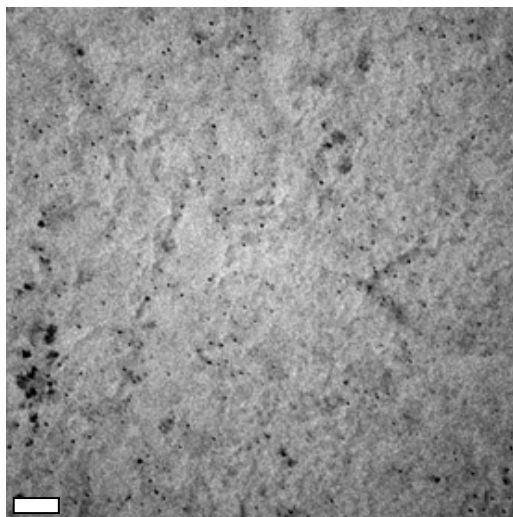
**3.3.1. TEM.** The metallic nature of the cobalt NP is clearly seen due to its high electron density (they appear thick black in conventional bright field TEM) and to its crystallinity (they appear bright white when observed in dark-field). Different features were observed on the grid: metallic particles embedded in an organic (peptides) pocket, large aggregates, individual NP, and combinations of these.



**Figure 1.** TEM images of Co NP synthesized in reaction 1 in the presence of peptide and DMF after a few minutes dispersed in water. Particles appear black in bright-field (left panel) and white in dark-field (right panel) images. Both images correspond to the same area. Bar, 100 nm.

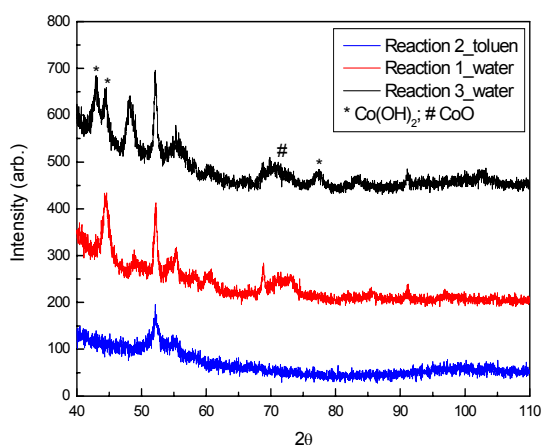
In reaction 1 after 15 min of the reaction, cobalt NP could be observed inside a pocket of organic material (Figure 1). When the reaction was left refluxing for 90 min, the number of large aggregates increases and there were more monodisperse NP (Figure 2). In reaction 2, we observed formation of NP which would indicate that DMF has a mild capping activity. This also suggests that amide groups can interact with cobalt metallic surfaces, but not very efficiently. Indeed, carboxyl and primary amine groups have been used as effective capping ligands for cobalt [4, 9, 10]. In reaction 3, when only peptide was present, there was only a small fraction of NP observed. In reaction 4, in the absence of any capping ligand (peptide

or DMF), the product is micro-sized magnetic precipitate that rapidly forms grey CoO in the presence of air and fluffy  $\text{Co}(\text{OH})_2$  with light pinkish colored supernatant in the presence of water.

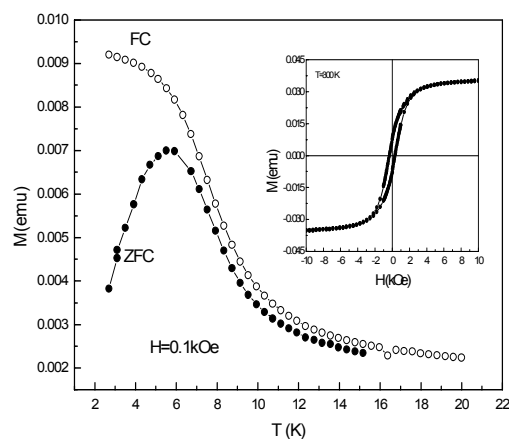


**Figure 2.** TEM images of Co NP dispersed in water (reaction 1, 90 min refluxing). Bar, 100 nm.

3.3.2. XRD revealed the typical diffraction patterns of mixtures of metal isoenergetic phases (hcp, fcc and epsilon) of Co nanoparticles (Figure 3, bottom line). Different crystal patterns are obtained at the same temperature, pressure and concentrations of  $\text{Co}_2(\text{CO})_8$  when changing the capping ligands [11] and are attributed to the surface stabilization and binding of the ligand to the growth of crystal. The broadness of the peaks is due to the nanometric size. However, when the samples were dispersed in water, the CoO (#) and  $\text{Co}(\text{OH})_2$  (\*) formed (Figure 3, middle and top line).



**Figure 3.** XRD patterns of synthesised materials from different reactions, using wave length of 1.798 Å from  $\text{Co K}_\alpha$  radiation.



**Figure 4.** Characterization of sample from reaction 1 by SQUID.

**3.3.3. SQUID magnetometry.** Analysis of the products of reactions shows a peak at around 6 K in the ZFC curve (Figure 4). This is an evidence for the presence of very small size NP (less than ~5 nm in diameter) in the sample. Up to room temperature (300 K), we still observed the splitting of the ZFC and FC curves and an open hysteresis loop with the coercivity  $H_c$  of about 0.33 kOe (inset of Figure 4), which would indicate that the sample contains also larger sized NP with a wide size distribution

**3.4. The role of ligand shell.** The thermal decomposition of cobalt carbonyl at high temperatures is a rapid phenomenon that produces a large amount of Co atoms in solution. These may form clusters [12] or complexes, with the clusters fusing or growing to form NP. The latter may go on to form microparticles. The critical nucleation radius is determined by the environment, which therefore in turn determines the efficiency of the formation of cluster versus the formation of the complexes. The growth of NP, the crystal structure and the cessation of the growth before the formation of microparticles also depend on the environment and are fundamentally regulated by the ligands. Thus, in the absence of ligand, particle surfaces are exposed, so they fused together and precipitated.

Co NP were formed in the presence of DMF alone (reaction 2), indicating that DMF acts as a capping ligand. In the presence of peptide alone (reaction 3) NP were produced less efficiently than in the presence of just DMF. This probably reflects the poor solubility of peptide in toluene. Moreover, peptide that does dissolve in toluene may process extended peptide-peptide interactions in the solvent to satisfy its extensive H-bonding requirements. Consequently, it may not function effectively as a capping ligand.

In peptide and DMF, the product of the reaction is similar to that observed in DMF alone. The major differences are observed after dispersing the reaction product in water. The stability of the material produced in the presence of only DMF is much reduced and quickly reacts with  $O_2$  and  $H_2O$  and precipitates. This observation is consistent with the known relationship between solvent polarity and complexation of Co atoms. Thus, when a solvent has a polarity index greater than 4 (equivalent of that of n-propyl alcohol), the decomposition of cobalt carbonyl releases cobalt atoms into solution, which are immediately coordinated by the solvent to form complexes and less particles are formed [4]. As long as the solvent polarity is low and the concentration of the ligand is not more than 100 times the precursor, NP are nucleated. Depending on the concentrations and ligands, different sizes, shapes and crystal structures can be nucleated and grown. The ligand will also determine the solubility (stability against aggregate) and chemical stability (against oxidation, hydroxylation among others).

#### **4. Conclusions**

NP were formed in the presence of peptide with some degrees of stability. From this starting point, the synthesis protocols may be optimized for particles with different sizes and shapes. The choice of capping ligands will be determined as a function of the stability of the NP, the monodispersity and the bio-molecular processes in which cobalt NP are to be used. The results open up the possibility of producing a bio-conjugate with the Co NP, which is designed for a particular application.

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