Hemoglobin loaded polymeric nanoparticles: Preparation and characterizations

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A R T I C L E   I N F O

Article history:
Received 14 January 2011
Received in revised form 8 March 2011
Accepted 23 March 2011
Available online 5 April 2011

Keywords:
Polymeric nanoparticles
Blood substitutes
Biocompatible polymers
Injectable systems

A B S T R A C T

In the present work polymeric nanoparticles based on Poly (maleic anhydride-alt-butyl vinyl ether) 5% grafted with m-PEG (2000) and 95% grafted with 2-methoxyethanol (VAM41-PEG) were loaded with human hemoglobin (Hb) and characterized from a physicochemical point of view. The assessment of structural and functional features of the loaded Hb was performed and the effect of the introduction of different reducing agents as aimed at minimizing Hb oxidation during the nanoparticles formulation process, was also investigated. Nanoparticles possessing an average diameter of 138 ± 10 nm and physico-chemical features suitable for this kind of application were successfully obtained. Although the oxidation of the protein was not avoided during its loading into nanoparticles, the presence of acid moieties in the polymeric structure is proposed to be directly involved in the protein inactivation mechanism.

1. Introduction

Medical interest in developing artificial oxygen carriers to be used as blood substitutes is stemming from the many limitations related to the commonly used blood transfusion. It is well known that collected blood samples present numerous drawbacks connected to time storage, timely availability and allogenic properties. Moreover, although current blood supply is safer than ever thanks to improved donor screening techniques and samples collection conditions, the diseases associated with blood transfusion can always occur due mainly to new viruses occurrence and eventually reduction of blood donors.

The potential benefits of developing artificial blood substitutes can be related to the universal compatibility, the on need availability, freedom from disease transmission, and long-term storage potential.

In the past decades, many strategies based on hemoglobin (Hb) and fluorocarbons have been investigated in order to obtain artificial oxygen carriers that could solve the above mentioned donor transfusion associated drawbacks.

Among Hb based cellular systems, the use of polymeric nanoparticles as artificial oxygen carriers presents many advantages mainly related to the feasibility of co-encapsulating reducing agents that can avoid Hb oxidation during the formulation process, and to the feasibility of easily modifying polymeric nanoparticles surfaces with antiopsonizing moieties that can impart Hb-loaded nanoparticles stealth properties (Piras et al., 2008). Avoiding Hb oxidation during nanoparticles preparation is a key issue in developing Hb loaded polymeric nanoparticles. The introduction of different reducing agents into Hb loaded nanoparticles formulation processes has been widely reported in literature; among these catalase, superoxide dismutase, peroxidase (Wright et al., 1996; Teramura et al., 2003; Napolitano, 2009), ascorbic acid, glutathione and methylene blue (Sakai et al., 1994; Sachdeva et al., 2003; Malchesky, 2010) can be cited.

Poly (maleic anhydride-alt-butyl vinyl ether) 5% grafted with m-PEG (2000) and 95% grafted with 2-methoxyethanol (VAM41-PEG) is an amphiphilic synthetic polymer which has shown favorable physical–chemical properties in the formulation of bioerodible polymeric nanostructured systems for the controlled release of high and low molecular weight active agents (Chiellini et al., 2008a,b; Piras et al., 2008). Each repeating unit of the hemiesters carries a hydrophilic ionizable acid group, a hydrophobic but potentially degradable ester residue, and an alkyl ether moiety. The polymer water solubility gradually increases under physiological conditions, thanks to the progressive carboxyl ionization and to an autocatalytic or enzyme mediated ester hydrolysis (Woodruff et al., 1972; Vert et al., 1992). Furthermore, the presence of PEG moieties eventually grafted onto the polymer matrix confers to the functionalised system stealth properties.

The present study is aimed at investigating the maintenance of Hb structural and functional features after its loading into...
VAM41-PEG based nanoparticles. The introduction of different reducing agents into the formulation system were also investigated in order to prevent Hb oxidation.

2. Materials and experimental procedures

2.1. Materials

Sodium dithionite, Drabkin’s reagent, potassium ferricyanide, m-PEG (2000), methylene blue, ascorbic acid and citric acid were purchased from Sigma Aldrich (IT). Poly (maleic anhydride-alt-buty1 vinyl ether) 5% grafted with m-PEG (2000) and 95% grafted with 2-methoxyethanol (VAM41-PEG) (MW 70 kDa) (Fig. 1) and O-glycidoxypropylidenglycerol grafted β-cyclodextrin (GIG–βCD) were synthesized at Biolab Laboratory (University of Pisa, Pisa, Italy).

Human serum albumin (25% in solution, B05AA01) (HSA) and human hemoglobin (5% w/v in Tris buffer pH 8, under the oxy form) (Hb) were kindly supplied by Kedrion S.p.A., Castelvecchio Pascoli, Lucca, Italy. Solvents and salts were obtained from Carlo Erba, Italy. Trizma buffer was purchased from BioChemika (IT). Spectra/Por dialysis membranes in cellulose esters and regenerated cellulose were purchased from Spectrum Labs.

Physiological solution was prepared by dissolving 9.0 g of NaCl in 1 liter of distilled water. The solution was sterilized in autoclave (121 °C for 20 min) before use and storage.

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Phosphate buffer saline solution (PBS 10 ×) was prepared by dissolving 2.0 g of KCl, 2.0 g of KH₂PO₄·H₂O, 80 g of NaCl, and 15.6 g of Na₂HPO₄·12H₂O in 1 liter of distilled water. The pH was adjusted to 7.4 with 10 N NaOH and the resulting solution was sterilized in autoclave (121 °C for 20 min) before use and storage. Working solutions were obtained by diluting PBS 10 × with distilled water either at 1:10 v/v (PBS 1 ×) or 1:100 v/v (PBS 0.1 ×); 50 mM Tris buffer solution was prepared by dissolving 7.09 g of Trizma buffer substance in 1 liter of distilled water. The pH was adjusted to 8.0 and the resulting solution was sterilized in autoclave (121 °C for 20 min) before use and storage.

2.2. Nanoparticle formulations

Hb loaded nanoparticles were prepared by means of the co-precipitation technique (Carlsson et al., 1998a,b) using VAM41-PEG as polymeric matrix and following a general proprietary procedure. The drop-wise addition of a water miscible polymeric organic solution to a water solution containing GIG–βCD and Hb, was performed under nitrogen atmosphere at 4 °C with a rate of 30 drops/min and a syringe equipped with a 22G needle. Once the addition of the polymer solution was completed, the resulting dispersion was kept under magnetic stirring for 30 min at room temperature and stored at 4 °C.

In the preparation of Hb loaded VAM41-PEG nanoparticles, the water solution consisted of 120 mg of GIG–βCD dissolved in 2.6 ml of deionised water and 0.2 ml of Hb solution. The polymeric solution was prepared by dissolving 50 mg of VAM41-PEG in 2.0 ml of 4:1 ethanol/water (VAM41-PEG solution).

VAM41-PEG nanoparticles loaded with Hb and citric acid were prepared by dropping a 2 ml of VAM41-PEG solution, in a 2.8 ml water solution containing citric acid at pH 6, 120 mg of GIG–βCD and 0.2 ml Hb solution. Five different citric acid concentration were tested: 1, 2, 3, 4 and 5 mM. The 5 mM citric acid formulation was filtered (5 μm) before submitting it to S.E.M. morphological analysis.

In VAM41-PEG Hb/HSA loaded nanoparticle formulations, 2 ml of VAM41-PEG solution was dropped in a 2.8 ml water solution containing 120 mg of GIG–βCD and different Hb/HSA concentrations (Table 1).

**Table 1** Details and diameter distribution of VAM41-PEG Hb/HSA loaded nanoparticles.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Hb mg</th>
<th>HSA mg</th>
<th>Nanoparticles average diameter (nm)</th>
<th>Average diameter standard deviation (nm)</th>
<th>Nanoparticles diameters distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb/HSA_1</td>
<td>5</td>
<td>5</td>
<td>78</td>
<td>40</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Hb/HSA_2</td>
<td>10</td>
<td>10</td>
<td>77</td>
<td>46</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Hb/HSA_3</td>
<td>2.5</td>
<td>7.5</td>
<td>78</td>
<td>45</td>
<td>Bimodal</td>
</tr>
<tr>
<td>Hb/HSA_4</td>
<td>10</td>
<td>50</td>
<td>10,000</td>
<td>–</td>
<td>Polymodal</td>
</tr>
</tbody>
</table>

Sodium dithionite solution (100 mM) was prepared by adding 174 mg of sodium dithionite to 10 ml of 0.1 × PBS under nitrogen atmosphere.

Carbon monoxide saturated solutions were prepared by bubbling an humidified carbon monoxide flux into PBS 1 × solutions for 30 min.

The commercial products were used without any preliminary purification if not otherwise stated.

![Fig. 1. VAM41-PEG chemical structure.](image-url)
In VAM41-PEG Hb/ascorbic acid/methylene blue loaded nanoparticles formulation, 2 ml of VAM41-PEG solution were dropped in a 2.8 ml water solution containing 120 mg of GIG–pCD, 0.2 ml of Hb solution and 33 µl of an ascorbic acid/methylene blue solution. The ascorbic acid/methylene blue solution was prepared by adding 360 µmol of ascorbic acid and 0.21 µmol of methylene blue to 1 ml of deionised H2O. All nanoparticles formulations were performed in triplicates.

2.3. Nanoparticles characterization

Dimensional analyses were carried out by means of Coulter LS230 Laser Diffraction Particle Size Analyzer, equipped with small volume module plus. Nanoparticles suspensions were added into the cell until 30–50% obscuration of PIDS detector was reached. Deionised water was used as background and diameter distribution was processed using Fraunhofer optical model. Three runs were performed on each sample.

Nanoparticles morphology was investigated by means of scanning electron microscopy (SEM). The samples were prepared from the purified and lyophilized nanoparticles. Gold sputtering was performed before SEM analysis.

Zeta potential analyses were carried out by using a Coulter Beckman Delsa 440SX at 25 °C with a 0.4 °C tolerance between upper and lower sensors. Nanoparticles suspensions were purified by centrifugation in ALC® PK 121R at 8000 g for 30 min, at 4 °C. Nanoparticles pellets were re-dispersed in physiological solution and diluted to a final concentration of 0.1 mg/ml. pH was adjusted to 5–5.5. Zeta potential values were calculated as the mean value of at least 10 replicates for each nanoparticles formulation.

2.4. Methemoglobin production

A 20 ml methemoglobin solution was generated by adding 0.78 mg of K3FeCN4 to a 20 ml water solution containing 0.6 µmol of Hb. The reaction was monitored by means of UV–vis spectra analysis.

2.5. Drabkin’s assay

Hemoglobin encapsulation efficacy (EE) and loading (Ldg) in VAM41-PEG nanoparticles was evaluated by a colorimetric assay based on Drabkin reagent. Experiments were carried out on purified nanoparticles collected after the centrifugation of 6 aliquots of 1 ml of nanoparticles suspensions at 8000 g for 30 min at 4 °C in ALC® PK121R centrifuge, AM–21 rotor. Once free from the supernatant, three of the collected pellets were suspended in 1 ml of Tris buffer each and pH adjusted to 8 with few microliters of 10 N NaOH. One hundred microliters of sodium dithionite solution were added to the nanoparticles solution under nitrogen atmosphere in order to reduce Hb. Progressive known amounts of carbon monoxide saturated solution were anaerobically added to the deoxygenated nanoparticles solution, in the absence of a gas phase. The ligation state of Hb was monitored by UV–vis analysis.

2.6. Hb characterizations

2.6.1. Evaluation of hemoglobin capability of reversibly binding oxygen

Hb capability of reversibly binding oxygen was detected by analysing changes in Hb UV absorption spectra (200–700 nm) in oxygenated and deoxygenated solutions. Oxygenated solutions were generated by means of a 10 min atmosphere nitrogen exposure, while deoxygenated solutions were obtained by means of a 10 min nitrogen flow exposure. UV spectra analysis was performed on dissolved nanoparticles formulations and on Solution 1 and Solution 2; nanoparticles dissolution were obtained by increasing formulations pH value up to 8, under magnetic stirring, using NaOH 10 N. Solution 1 was prepared by adding 8.5 mg of VAM41-PEG dissolved in 400 µl of ethanol, to a 10 ml of a water solution containing 3 mg of Hb and 8.5 mg of GIG–pCD. Solution 2 was obtained by mixing 3 mg of Hb and 3 mg of HSA in 10 ml of deionised water.

2.6.2. CO-saturation curve

Hb loaded nanoparticles samples were prepared by centrifuging 3 ml nanoparticles aliquots at 8000 g for 30 min at 4 °C. Resulting pellets were suspended in 3 ml of Tris buffer and pH adjusted to 8 with few microliters of 10 N NaOH. One hundred microliters of the sodium dithionite solution were added to the nanoparticles solution under nitrogen atmosphere in order to reduce Hb. Progressive known amounts of carbon monoxide saturated solution were anaerobically added to the deoxygenated nanoparticles solution, in the absence of a gas phase. The ligation state of Hb was monitored by UV–vis analysis.

2.6.3. Hill coefficient determination

Hill plots were performed in the usual way by plotting Log(Y) / (1–Y) vs. Log [CO], where Y is the fraction of Hb ligand binding sites filled and CO are carbon monoxide moles added to the Hb solution. The Hill coefficient nH was obtained as the slope of the straight line that fits the central part of the Hill plot (0.25 < Y < 0.75). Log [CO]50 values were obtained from the intercept of the Hill plot with the axis of the abscissae. Y values were obtained from the UV–vis spectra using absorbance values at 419 and 430 nm according to the Beutler and West method (Beutler and West, 1984). Hill coefficient estimation was calculated as the mean value of three replicates.

2.7. Instrumentation

UV–vis absorption spectra measurements were performed by Jasco V–530 and UNICAM UV 500 ThermoSpectrornic spectrophotometers.

UV circular dichroism spectra were performed by means of Jasco J–715 spectropolarimeter.

3. Results and discussion

3.1. VAM41-PEG nanoparticles loaded with Hb

The preparation of biocompatible polymeric nanoparticles loaded with Hb to be used as artificial oxygen carriers was performed by means of the co-precipitation technique. The process
is based on the drop-wise addition of a water–miscible organic solvents solution of the synthetic polymer into an aqueous protein solution, under gentle magnetic stirring. During the co-precipitation process, the polymeric material gives rise to microphase separation because of its low water solubility; concurrent interaction with protein molecules leads to nanoparticles formation.

The co-precipitation of the polymer was performed using O-glycidyl-O-isopropylidenglycerol grafted β-cyclodextrin (GIG-β-CD) (Chiellini et al., 1991) as a stabilizer in the aqueous phase. Cyclodextrins are commonly used as amphiphilic stabilizers due to their properties of complexing hydrophobic molecules. Cyclodextrins posses a typical “basket” structure, with an interior part which is commonly less hydrophilic than the aqueous environment and thus able to host other hydrophobic molecules. In contrast, the exterior is sufficiently hydrophilic to impart cyclodextrins water solubility.

In the production of Hb loaded nanoparticles controlled conditions (4 °C, nitrogen atmosphere) were used in order to reduce or minimize oxidative processes that can lead to methemoglobin (Met-Hb) or Ferryl-Hb formation.

VAM41-PEG based human Hb loaded nanoparticles were obtained possessing an average diameter of 138 ± 10 nm and a monomodal distribution; a uniform and spherical shape was observed by means of scanning electron microscopy (SEM). Zeta potential analysis performed on nanoparticles suspension showed an average value of −5.40 ± 0.15 mV; this value is associated to the exposure of PEG brushes onto nanoparticles surface, which promotes the achievement of an anti-sonozing effect in vivo as discussed in previous studies (Piras et al., 2008). Hb EE and Ldg were estimated to be 20 ± 3% and 15 ± 3%, respectively.

3.2. Characterization of loaded Hb

Analysis devoted to investigate the chemical–physical properties of Hb loaded inside VAM41-PEG nanoparticles were carried out.

To check whether Hb maintained functional properties after its loading into VAM41-PEG nanoparticles, i.e. the protein ability of reversibly binding oxygen, UV–vis absorbance spectra analysis were performed on dissolved VAM41-PEG Hb loaded nanoparticles. As reported in Fig. 2 the obtained spectrum showed absorbance peaks approximately at wavelengths typical of Ferryl-Hb (Fe4+) (418, 545, 580 nm), while the absence of a peak at 630 nm excluded the formation of relevant amounts of Met-Hb (Fe3+).

These data suggest that an oxidation process, probably occurring during nanoparticles preparation, might lead to the oxidation of Deoxy-Hb to Ferryl-Hb.

To check whether this oxidation process affects also the maintenance of the protein secondary structure, circular dichroism (CD) spectra were measured and compared with the native Hb one.

As shown in Fig. 3 Hb loaded inside VAM41-PEG nanoparticles showed α-helix absorbance peaks at 208 and 222 nm analogous to that of native Hb.

The discrepancy between the two spectra (wavelength >220 nm) may be associated to the oxidation of the Hb during nanoparticles preparation and the consequent formation of Ferryl-Hb, as already ascertained by UV–vis analysis. It is in fact known that oxidative processes, especially those involving reactive oxygen species, may lead to Hb aminoacids oxidation (in particular βCys-93, βCys-112 and βMet-55 are irreversibly oxidized to cysteic acid and methionine sulfoxide) resulting thus into alterations of Hb CD spectrum (Jia et al., 2007).

As a consequence of this oxidation process, the protein looses its ability to bind reversibly molecular oxygen. Indeed, the UV–vis spectrum was unchanged in oxygenated and deoxygenated solutions, even after prolonged N2 or O2 exposure. To circumvent this problem we firstly added different reducing or protective agents into nanoparticles formulation process.

3.3. VAM41-PEG Hb/citric acid loaded nanoparticles

Citric acid is an effective antioxidant and radical-scavenger agent. The antiradical properties of citric acid are represented mainly by its reaction with hydroxyl radicals, while the antioxidant activity of the acid is due instead to its reactions both with molecular oxygen and with hydrogen peroxide (Gromovaya et al., 2002).

To investigate whether the introduction of citric acid into nanoparticles formulation system could avoid Hb oxidation, different citrate buffer concentrations were tested (1, 2, 3, 4 and 5 mM).

Nanoparticles dimensional analysis showed the feasibility of producing nanoparticles possessing a small diameter and a monomodal distribution in presence of the reducing agent up to a citrate buffer concentration of 2 mM (Table 2).

Moreover it was observed that higher concentrations of citrate buffer (i.e. 5 mM) determine the precipitation of Hb in the aqueous phase, before polymer dropping.

![Fig. 2. Hb loaded VAM41-PEG nanoparticles UV–vis spectra analysis.](image)

![Fig. 3. Hb loaded VAM41-PEG nanoparticles CD spectra analysis (red curve), native Hb CD spectrum (Jia et al., 2007) (black curve). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
As reported in Fig. 4, morphological analysis showed a spherical and homogeneous morphology for all of the citrate buffer concentrations tested.

To check whether the introduction of Citrate buffer avoids Hb oxidation during nanoparticles formulation process, UV–vis spectra analyses have been performed on the prepared citrated samples once dissolved in alkaline solution. As reported in Fig. 5 the Hb absorption spectrum posses 418, 545 and 580 nm absorbance peaks in all of the five tested formulations.

Table 2
Citrate buffer Hb-nanoparticles diameter distribution.

<table>
<thead>
<tr>
<th>Citrate buffer concentration (mM)</th>
<th>NPs dimension (nm)</th>
<th>Average diameter standard deviation (nm)</th>
<th>Nanoparticles diameters distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>8</td>
<td>Monomodal</td>
</tr>
<tr>
<td>2</td>
<td>80</td>
<td>5</td>
<td>Monomodal</td>
</tr>
<tr>
<td>3</td>
<td>154</td>
<td>–</td>
<td>Polymodal</td>
</tr>
<tr>
<td>4</td>
<td>108</td>
<td>–</td>
<td>Polymodal</td>
</tr>
<tr>
<td>5</td>
<td>10,000</td>
<td>–</td>
<td>Polymodal</td>
</tr>
</tbody>
</table>

These results suggest that the introduction of citrate buffer into the formulation system, although did not affect the possibility of obtaining Hb loaded nanoparticles possessing a nano-sized diameter distribution and a spherical and homogeneous morphology, did not avoid the oxidation process of Hb.
3.4. VAM41-PEG Hb/ascorbic acid/methylene blue loaded nanoparticles

The introduction of ascorbic acid/methylene blue in the nanoparticle formulations was performed in order to check whether the activity of these reducing agents could be able to avoid or minimize Hb oxidation.

Methylene blue is a heterocyclic aromatic compound with molecular formula C$_{16}$H$_{18}$N$_3$SCl and is commonly used as an agent for the treatment of drug-induced MethHemia and in patients with congenital MetHemia. Methylene blue acts as a cofactor for the NADPH cytochrome b5 Met-Hb reductase enzyme (Yazbeck-Karam et al., 2004; Ying Wang et al., 2009). It can also be reduced in red blood cells and presumably in tissues to leucomethylene. Leucomethylene reacts spontaneously with high concentrations of Met-Hb in blood, rapidly reducing Met-Hb to Hb, even in the presence of oxygen (Sachdeva et al., 2003).

Methylene blue is easily reduced to the leucomethylene by a variety of agents including ascorbic acid (Mowry and Ogren, 1999).

Since ascorbic acid, leucomethylene and Hb standard reduction potentials are respectively −0.77, 0.011 and 0.092 mV, Met-Hb conversion to Hb can be performed by leucomethylene, if methylene blue is constantly reduced by ascorbic acid.

According to the literature (Mowry and Ogren, 1999), leucomethylene solution was prepared by adding a methylene blue water solution to an ascorbic acid water solution thus obtaining a final methylene blue concentration of 0.05% with respect to ascorbic acid concentration. As above mentioned, ascorbic acid excess is necessary to reduce methylene blue to leucomethylene during Met-Hb reduction. The ability of reducing Met-Hb to Hb was confirmed by UV–vis spectroscopy on a Met-Hb solution before testing the leucomethylene/ascorbic acid system for the preparation of Hb loaded nanoparticles. Moreover the capability of preventing Hb oxidation was assessed on Hb solution, in the presence of oxygen (Ying Wang et al., 2009).

The combination of ascorbic acid and leucomethylene was then employed in the nanoparticles formulation process, by dropping the ethanol/water polymeric solution into a water solution containing the stabilizer, Hb and the reducing agents with an ascorbic acid/HEME ratio of 10:1. The obtained nanoparticles had an average diameter of 100 ± 16 nm and a monomodal distribution. The presence of Ferryl-Hb UV–vis absorbance peaks (545 and 580 nm) suggested that the introduction of ascorbic acid/leucomethylene into nanoparticle formulations seems not to avoid or reduce Hb oxidation (Fig. 6).

An additional amount of ascorbic acid/leucomethylene combination was added to the dissolved nanoparticles dispersion to check whether the reducing agents would be able to reduce Ferryl-Hb to Hb after the nanoparticles formulation process. As revealed by UV–vis absorbance spectrum analysis, ascorbic acid/leucomethylene could not reduce Ferryl-Hb even after the formulation process had taken place.

Further control experiments, performed with empty nanoparticles suspensions to which Hb was added after nanoparticles formation, showed both that the polymer and β-cyclodextrin do not interfere with ascorbic acid/leucomethylene capacity of reducing Met-Hb to Hb in solution, nor with the capability of Hb of reversibly binding oxygen after its addiction to the dissolved empty nanoparticles solution. This notwithstanding, the ascorbic acid/leucomethylene system was not able to prevent Hb oxidation during nanoparticles formation or even to reduce oxidized encapsulated Hb.

3.5. VAM41-PEG Hb/HSA loaded nanoparticles

Human serum albumin (HSA) is the most abundant plasma protein; it consists of a single polypeptide chain of 585 amino acids with a molecular weight of 66.5 kDa. It poses a molecular structure that can be depicted as a cylinder with polar outer walls and a hydrophobic central core (Ghuman et al., 2005; Michnik et al., 2005).

Thanks to this property HSA is widely used as a stabilizing component in pharmaceutical and biological products, such as vaccines, recombinant therapies and coatings for medical devices (Bosse et al., 2005). Different concentrations of HSA were added to the formulation bath of Hb loaded nanoparticles to stabilize Hb during nanoparticles formation.

UV–vis spectrum analysis have been performed on solution containing Hb and HSA (1:1 molar ratio) to check whether HSA could interfere with Hb activity of reversibly binding oxygen.

As reported in Fig. 7, Hb maintains its functional activity in presence of HSA. Absorption peaks at 415, 541 and 577 nm are present in the oxygenated solution, while 430 and 555 nm absorbance peaks are present in the deoxygenated solution.

Dimensional analysis showed the feasibility of producing Hb/HSA loaded nanoparticles up to 20 mg of total protein content in the formulation bath (formulation Hb/HSA; Table 1). UV–vis spectra analysis performed on the above mentioned dissolved formulations revealed that the introduction of HSA into the formulations seems not to avoid Hb oxidation. In fact, as reported in Fig. 8, typical UV–region Ferryl-Hb peaks (545 and 580 nm) are present in the absorption spectra of all Hb/HSA formulations; moreover, the presence of typical visible region Met-Hb peak (631 nm) was also observed in Hb/HSA_4 absorbance spectrum.

Although the formation of Met-Hb (Fe$^{3+}$) might be indicative of a lower Hb oxidation level, Hb/HSA_4 formulation cannot be taken into account for the production of Hb loaded nanoparticles due to
the presence of Met-Hb and to the large diameter distribution which was estimated to be around 10 µm as reported in Table 1.

The inability of the tested reducing agents to prevent Hb oxidation during its loading into VAM41-PEG polymeric nanoparticles, reveals that the oxidation is probably not the only event that leads to the protein inactivation. In particular, a possible correlation between VAM41-PEG carboxylic moieties exposure into nanoparticles microenvironment and the protein inactivation seems to be appropriate. As well stated by Kristinsson (2002), low pH conditions, or the contact of Hb with acidic membrane interfaces leads to a protein partial unfolding resulting in changes in the HEME pocket structure. The protein ability of binding oxygen is then inhibited and the protein pro-oxidative activity is enhanced. This phenomenon creates variations on Hb UV–vis and circular dichroism absorption spectra which appear to be comparable to our findings. Moreover the decrease in Hb oxidation in presence of high concentration of HSA is a confirmation of this hypothesis. In this case the HSA protective effect originates from the physical strong interaction that the protein has with VAM41-PEG polymer, as demonstrated elsewhere (Piras et al., 2008). The formation of such complexes reduces or prevents the direct contact between the polymer and Hb, decreasing the protein inactivation process. Although further investigations are needed to confirm the present work hypothesis, the observed protein functionality alterations once loaded into nanoparticles seem to be attributable to the acid microenvironment generated by VAM41-PEG carboxylic groups. Reasonably, the employment of polymers presenting acidic moieties may lead to similar results, suggesting this feature as unsuitable for polymers which are likely to be used for the production of Hb loaded nanoparticles.

3.6. Reduction of Hb loaded into VAM41-PEG nanoparticles and CO saturation curve

Surprisingly, anaerobic addition of a molar excess of sodium dithionite to previously deoxygenated suspensions of Hb-loaded VAM41-PEG nanoparticles did reduce, at least partially, the encapsulated Hb. This is shown in Fig. 9 where the appearance of the 555 nm peak of deoxyHb upon dithionite addition is clearly evident.

Although reduction is not complete (as shown by the presence of a peak at ~530 nm), the encapsulated Hb is now able to bind the CO ligand, as demonstrated by the spectrum in Fig. 9 obtained upon addition of molar excess of CO.

Interestingly, this last spectrum shows also an absorption decrease at 545 nm and only a slightly increased absorption at 630 nm, suggesting that the presence of dithionite + CO is effective in reducing the Ferryl-Hb present without causing excessive formation of Met-Hb.

The findings reported in Fig. 9 open the possibility of testing the functional properties of encapsulated Hb via the CO-saturation curves. Results are shown in Fig. 10 under the form of the Hill plot: Log(Y/(1-Y)) vs. Log(CO moles).

The loss of cooperativity of encapsulated Hb may be due either to the fact that encapsulation within the nanoparticle matrix hinders the protein quaternary transition (upon deoxygenation) from the high affinity R-state to the low-affinity T-state (Levantino et al., 2003; Schirò and Cupane, 2007), or to the incomplete protein reduction that does not allow to reach a fully deoxygenated state. Further studies will be necessary to clarify this point.

4. Conclusions

The present work demonstrated the feasibility of loading Hb into VAM41-PEG based polymeric nanoparticles with good efficiency. Hb undergoes inactivation processes during nanoparticles formulation, which lead to the generation of Met-Hb and Ferryl Hb and as a consequence inhibits the protein ability of reversibly binding oxygen. Although different reducing agents were introduced into the tested formulations in order to prevent the formation of inactive Hb species, the protein functional features were
not maintained. One of the most significant findings raising from this study is that the presence of acidic moieties into the polymer structure is likely the main cause of protein inactivation. On the other hand, the presence of dithionite + CO seems to protect Hb against Ferryl Hb formation. In order to improve the design of artificial oxygen carriers based on polymeric acidic matrices, the introduction of carboxy-Hb (Sakai et al., 2008; Agashe et al., 2010) together with a preserving agent such as HSA opens a new route to avoid Hb oxidation during nanoparticles preparation.

References