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PROTEIN DISULFIDE ISOMERASE-ASSISTED REFOLDING OF RIBONUCLEASE A NANOPARTICLES

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Protein disulfide Isomerase (PDI), Ribonuclease A (RNase A), Sonochemistry, Nanoparticles, Protein Refolding, Enzymatic Activity

ABSTRACT
This work highlights the refolding of ribonuclease A (RNase A) as a new methodology to recover the biological function of protein stabilized as nanoparticles, assisted by protein disulfide Isomerase (PDI).We have shown that RNase A nanoparticles loose 35% of enzymatic activity when compared to the native RNase A, and PDI was able to restore it. Moreover, the application of PDI on nanoparticles suspension, in the presence of appropriate oxidative environment, suggests that RNase A nanoparticles may refold into the aqueous medium by a PDI induced-structural change. In this way, this work demonstrates the potential of using RNase A nanoparticles as advanced bio-functional materials for diverse applications such as drug delivery.

INTRODUCTION
Early in 1990, Suslick and co-workers have developed a method for the synthesis of proteinaceous microspheres filled with water-insoluble liquids (Suslick and Grinstaff 1990; Gedanken 2008). These microspheres are produced by means of ultra-sonication of a two-phase starting solution that consists of a protein aqueous solution and an organic solvent.
Numerous works report on the development, characterization and application of nanoparticles created by means of ultrasonication.

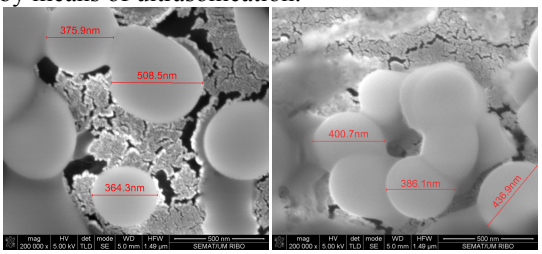

Figure 1- SEM images of RNase A nanoparticles and the sizes of these particles.

However, the mechanistic insights for its formation, the structural changes, and the folding process they suffer during sonication are not fully understood. Enzyme based microspheres constitute an interesting approach for this issue since their conformational structure can be predicted with the study of their catalytic activity after sonication.
Several studies have showed that enzyme based microspheres prepared by ultrasonication are still catalytically active and the microspherization process, unlike denaturation in which the protein's biological activity is destroyed, reduces its biological activity but does not destroy the active sites of the enzymes' nanoparticles (Avivi and Gedanken 2005; Avivi and Gedanken 2007). In this work, RNase A protein nanoparticles were synthesized and the catalytic ability of protein disulfide isomerase (PDI) to recover its activity, as a function of the redox environment, was assessed and compared to the native enzyme.
PDI is an enzyme that is expressed on endoplasmic reticulum and possesses three major activities: reduction, oxidation, and isomerization (shuffling) of disulfide bonds in substrate proteins, depending on the PDI's active site state (Lyles and Gilbert 1991; Wilkinson and Gilbert 2004). The probable explanation for the conformational changes that occur in the proteins subjected to ultrasonic radiation is presented in this work, by means of PDI–assisted refolding catalytic studies, and by nanoparticles FTIR spectra analysis.

RESULTS
A group of parameters were determined to characterize the nanoparticles samples: morphology, particle size, polidispersity index and zeta potential. Sonochemically prepared RNase A nanoparticles presented spherical morphology (Figure 1) with sizes ranging from 400nm to 1000 nm and a polidispersity near 1 and a zeta potential of -20 ± 3 mV. This indicates that the nanoparticles formulation are reasonably stable.

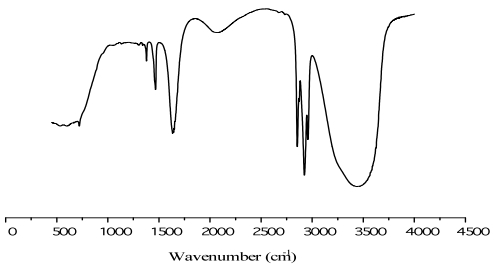

Figure 2- Infrared spectra of RNase A nanoparticles.

In order to study the conformational changes that occur on proteins during sonication A FTIR study was

perfomed on RNase A nanoparticles. It was found that they possess a typical protein like spectra on FTIR (Figure 2) which may indicate that the secondary structure is not involved on the nanoparticles formation. To corroborate this findings a catalytic study on RNase A nanoparticles was performed. First, it was observed that RNase A nanoparticles only loose 35% of enzymatic activity when compared to the native RNase A (Table 2).

Table 2- Activity and Protein data on freeze-dried RNase A nanoparticles and native RNAse A.

RNase A	Activity U/mg	Protein mg/L
Freeze-dried nanoparticles	3.56 ± 0.016	16.02 ± 0.002
Native	5.49 ± 0.035	134.59 ± 0.005

Further application of PDI together with its cofactor (Glutathione buffer - GSSG/GSH) was able to restore this lost activity (Figure 3). The Glutathione buffer is an important factor to set PDI's active site on the reduced form to act as an isomerase and act upon disulfide bonds of RNase nanoparticles.

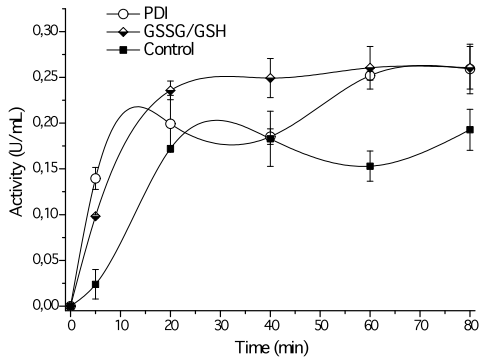

Figure 3- Time-course reaction of the oxidative folding of RNase A lyophilized nanoparticles in the presence of: 1)PDI with GSSG/GSH buffer, 2) GSSG/GSH buffer and the 3) control with 0,1 M Phosphate buffer pH 7.5.

Moreover, the study perfomed on suspension nanoparticles suggest that PDI together with both redox buffers (GSSG/GSH and DTT) are able to induce the increase of protein content in the aqueous medium (Figure 5). This can be further studied for the possibility of nanoparticles to open by the mentioned refolding mechanism, in the presence of an appropriate buffer and PDI. In this way, this work demonstrates the potential of using RNase A nanoparticles together with PDI as advanced bio-functional materials for diverse applications such as drug delivery.

CONCLUSIONS

This study allowed the investigation of the probable explanation for the conformational changes that occur in the proteins subjected to ultrasonic radiation, by means of FTIR spectra analysis on RNase A nanoparticles and catalytically study. The main conclusion is that secondary structure does not seem to be involved in nanoparticles formation and it is conserved after nanoparticles development. Application of PDI was crucial to attain this conclusion because both PDI and Gluthatione redox buffer were able to refold nanoparticles towards a catalytically active state.

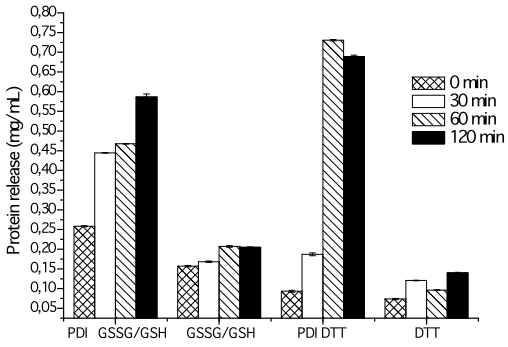

Figure 5- Protein assessment for refolding studies on aqueous phase of nanoparticles solution after treatment with refolding buffers: 1) PDI with GSSG/GSH buffer, 2) GSSG/GSH buffer, 3)PDI with DTT and 4) DTT.

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