Title
Enzyme Nanocapsules as Intracellular Scarvagers for Reactive Oxygen Species

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Enzyme Nanocapsules as Intracellular Scarvagers for Reactive Oxygen Species

A thesis submitted in partial satisfaction

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by

Shuoran Li

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ABSTRACT OF THE THESIS

Enzyme Nanocapsules as Intracellular Scarvagers

for Reactive Oxygen Species

by

Shuoran Li

Master of Science in Biomedical Engineering

University of California, Los Angeles, 2012

Professor Tatiana Segura, Co-chair

Professor Yunfeng Lu, Co-chair

We use superoxide and catalase synergy nanocapsule system to reduce the cytotoxicity caused by superoxide radicals, a typical reactive oxygen species (ROS). Enzymes are encapsulated inside positive shells via APS/TEMED initiated polymerization. The diameter and charge of the non-degradable nanocapsules are suitable for intracellular delivery and clear endocytosis has been observed via fluorescence microscope. Under induced radical environment, higher cell viability is observed when cells are incubated with both nanocapsules at the ratio of nSOD/nCatalase=5 compared with only nSOD or nCatalase system. We believe that the use of this synergy delivery providing a sound progress for eliminating ROS *in vitro* via synergy protein therapy. What's more, this bi-enzyme nanocapsule system can also be seen as solid foundation for multi-enzyme nanocapsule system to achieve other complex therapeutic applications.

The thesis of Shuoran Li is approved.

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University of California, Los Angeles

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1 Introduction

Free radicals exist in all biological system and have long been studied[1]. Free radical theory of aging[2, 3] have made it clear that free radical reactions, arising during the process of normal metabolism, are responsible for the progressive accumulation of changes with time that are associated with or responsible for the ever-increasing likelihood of disease and death while age advances.

They are many diseases that can be caused by free radicals, such as cancers[4-6]. It is believed that some endogenous free radicals can be result in tumor formation by serving as a continuous source of tumor initiators and promoters[2, 5, 7]. There have been many studies showing that free radical scavengers can prolong survival in various cancers, such as gastric cancer[8] and colonic cancer[9]. What's more, a number of antioxidants[10, 11]show positive effects in model cancer system. Free radicals can also cause atherosclerosis[6, 12]. Free radical can actually cause the cholesterol accumulation[13] , which leads to atherosclerosis in the end. Free radical scavengers are also being used to promote the regression of atherosclerosis[14] and suppress oxidative stress-induced endothelial damage and early atherosclerosis[15]. Besides cancer and atherosclerosis, free radicals can also lead many other diseases like essential hypertension, senile dementia of the Alzheimer type, amyloidosis, immune deficiency of age[6].

In this paper, we choose superoxide radical as our target eradicating radical species, because superoxide radical is one of the most dangerous endogenous toxicant[16], which produces lots of other cytotoxic compounds, such as hydroxyl radical, peroxynitrite, or hydrogen peroxide besides its direct toxic action[17]. What's more, many cellular pathways inside cells can lead to superoxide, which is the intracellular sink of radicals[18]. A variety of radicals, either directly or via reduced glutathione (GSH) as an intermediate, can transfer their unpaired electron to oxygen to give superoxide[18]. Superoxide radical can cause aging[2] and damage of organs[19-21], DNA chain breakage[22, 23] and is also related to

many other diseases, such as breast cancer[24], ocular inflammatory disease[25], Behcet's disease[26], Parkinson's disease[27] .

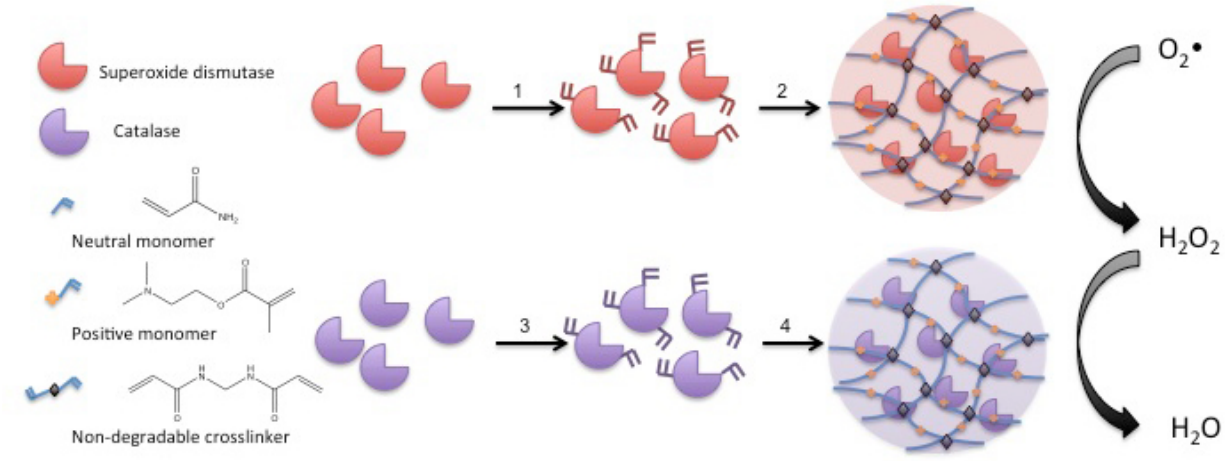
In order to effectively reduce superoxide radical, we have considered many antioxidants. However, most of those antioxidants are dietary small molecule like vitamin C, vitamin E, β-carotene, selenium[28], which have very limited effects on longevity [29]. What's more, the dosage of those antioxidants needs to be very strict, otherwise may cause toxicity[28] and perhaps even higher cancer risk[30]. Instead of using small-weight molecules, specific-functioned proteins are better choices because of their high catalyzing efficiency and modifiable structure based on different targeting needs.

In our research to eradicate the negative effects caused by superoxide radicals, we choose superoxide dismutase (SOD), which is an enzyme that catalyzes superoxide radicals[31]. The reaction catalyzed by SOD is $O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$. It has been proved that superoxide dismutase can delay neuronal apoptosis[32], relieve stress[33], diminish inflammation effects [34, 35] and selectively kill cancer cells[36]. In particular, a clear link has been identified between forms of amyotrophic lateral sclerosis and inactivation of SOD due to mutation[37]. There are also studies showing that cancer cells survival depending on the low SOD amount and can be eliminated when adjusting the SOD concentration[36].

However, hydrogen peroxide, the product of SOD usage, can still cause neuronal cell death[38] and damage of DNA[39]. Tumor cells produce great amount of hydrogen peroxide, which indicates the presence of hydrogen peroxide could also increase the mutation of the cells[40]. It could also induce the autoxidation of human red cell lipids[41].

In order to reduce the SOD toxicity by eliminating the formation of hydrogen peroxide, catalase and superoxide dismutase conjugates has been studied. The reaction of catalase in the decomposition of hydrogen peroxide is: $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$. The synergy effect of those two enzymes reduces the oxygen toxicity in great extent[42]. Promising life span increasing[43] and preventive DNA from breakage[44] has been observed. When conjugated to PEG, the enzyme conjugates actually increase endothelial enzyme activity[45].

After choosing SOD/ catalase as our experimental protein pair, the efficient delivery step into cells is our biggest challenge. Protein therapy[46], which delivers proteins into the cells to replace the mal-functioning protein, is a promising therapeutic. However, poor stability of proteins inside cells because of existence of proteases and low cellular permeability make the clinical applications of proteins rare[46, 47]. Although proteins can be transported into cells by receptor-mediated endocytosis[48], they can still be trapped inside endosome and degraded inside lysosome without functioning. Similarly, liposome-wrapped delivery is still at low efficiency level.[49, 50] Oppositely, the uses of cell-penetrating peptides (CPP) improve the delivery efficacy[51-53], yet still have no protection for proteins and thus don't increase the stability of proteins at all[54].



Scheme 1. Schematic showing the synthesis of cationic single-protein nanocapsules and their synergy effects in clearing superoxide radicals. 1, formation of polymerizable proteins by conjugating polymerizable acryl groups to the protein via reacting *N*-(3-aminopropyl)methacrylamide(APm) with carboxylic acid groups on SOD surface. **3,** formation of polymerizable proteins via reacting N-hydroxylsuccinimide ester (NAS) with amine groups on catalase surface. **2,4,** formation of non-degradable nanocapsules from N,N'-methylenebisacryl amide(BIS), acrylamide(AAm), 2-(dimethylamino)ethyl methacrylate(DMAEMA).

In our study, we utilize this SOD/Catalase system as a representative antioxidant system in getting rid of exogenous superoxide radical, which is induced by radical toxicant, such as paraquat[55, 56]. In order for this SOD/Catalase system to function in the most efficient way, we develop an effective bi-enzyme synergy system based on a novel intracellular system, which is composed of single-protein core and a thin

layer of shell[57]. This polymer can maintain enzymes' activities, protect them from being digested and thus enhance the gradual release to cytosol[57].

2 Materials and methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise noted, and were used as received. Catalase, from bovine liver (Cat.No.C30-100MG), is bought from Sigma-Aldrich. Superoxide dismutase is bought from Calzyme(Cat. No.110A3000).

2.2 Instruments

UV-Visible adsorption was acquired with a Beckman Coulter DU®730 life science UV/Vis Spectrophotometer. Zeta potential and particle size distribution were measured with a Malvern particle sizer Nano-ZS. Fluorescent images of cells were obtained with either Zeiss Axio Observer.Z1 fluorescence microscope. Fluorescence intensity and cell viability were measured by a Fujifilm BAS-5000 plate reader.

2.3 Methods

2.3.1 Single-protein encapsulation

2.3.1.1 nCatalase synthesis

For the synthesis of nCatalase, 1 mg native catalase was first dissolved into 1ml of 20 mM pH=7 phosphate buffer. Then N-hydroxylsuccinimide ester (NAS), prepared in a 100μg/μL anhydrous DMSO solution, was added into protein solution with incubation for 2 hrs at 4°C. The modified protein solution was then dialyzed overnight in 20 mM pH=7 phosphate buffer at 4°C. 4-dimethylaminoantipyrine(DMAAP), N,N'-methylenebisacryl amide(BIS), acrylamide(AAm), 2-(dimethylamino)ethyl methacrylate(DMAEMA) were added into the dialyzed protein solution sequentially with vortex. The molar ratio of protein/ NAS/ DMAAP/ BIS/ AAm/ DMAEMA was 1/150/1/1/4/2. Radical polymerization was initiated by adding both ammonium persulfate (1/10 molar ratio of total monomers) dissolved in deionized water and the same volume of 10% N,N,N',N'-tetramethylethylenediamine into reaction solution. The reaction was incubated for 2 hrs at 4°C.

2.3.1.2 nSOD synthesis

For the synthesis of nSOD, 0.2 mg SOD was first dissolved in 10mM pH=6 MES buffer to make the final protein concentration at 1 mg/ml. 0.8 mg *N*-(3-aminopropyl)methacrylamide(APm), 0.02mg N-hydroxylsuccinimide(NHS), which is dissolved in DMSO, and 0.4 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were added sequentially into the protein solution. The reaction was allowed for 2 hrs at 4°C. The modified protein solution was then dialyzed overnight in 20 mM pH=7 phosphate buffer at 4°C. N,N'-methylenebisacryl amide(BIS), acrylamide(AAm), 2-(dimethylamino)ethyl methacrylate(DMAEMA) were added into the dialyzed protein solution sequentially with vortex. The molar ratio of protein/ BIS/ AAm/ DMAEMA was 1/1/4/2. Radical polymerization was initiated by adding both ammonium persulfate (1/10 molar ratio of total monomers) dissolved in deionized water and the same volume of 10% N,N,N',N'-tetramethylethylenediamine into reaction solution. The reaction was incubated for 2 hrs at 4°C.

2.3.2 Characterization of nSOD and nCatalase

0.8% agarose gel was used in electrophoresis, which was taken place at 110V for 15 min. The hydrodynamic size distribution and Zeta Potential of protein nanocapsules were measured by a dynamic light scattering (DLS, Malvern particle sizer Nano-ZS).

2.3.3 Nanocapsule activity test

2.3.3.1 nSOD activity test

SOD activity test is pyrogallol[58] based. Pyrogallol, when in alkaline solution, absorbs oxygen from the air, turning purple from a colorless solution. 20 mM pyrogallol solution is made in degased DI water. Get the buffer solution of 50mM Tris- HCl with 1.5 mM EDTA solution (for getting rid of iron) and adjust the pH=8.2 ready for use. Using the buffer solution as blank at 420nm, and adding 7 μL pyrogallol solution into 693 μL buffer as the standard curve for 3 min under continuous spectrophotometric test mode. Then adding SOD samples (SOD final concentration around 0.01mg/ml) with 7 μL pyrogallol into buffer to make the final volume at 700 μL to get the test curve for 3 min. The slope of the curve/ slope of the standard curve is calculated as the inhibition rate. According to the assay paper[58], 100 ng/ml native

SOD can inhibit the pyrogallol reaction by 50%. But the concentration of SOD what we uses to get 50% may differ a little. Record the native SOD concentration at 50% inhibition. After native SOD test curve is confirmed, adjust nSOD concentrations in the test to make the test curve also reaches the 50% inhibition. The activity ratio equals to native SOD concentration divided by nSOD concentration, both at 50% inhibition.

2.3.3.2 nCatalase activity test

Dilute 0.7 μL 30% hydrogen peroxide into 699 μL pH=7，50mM phosphate buffer and make the absorption between 0.520 to 0.550 at 240 nm. Catalase solutions were immediately prepared before use and were diluted in 50mM phosphate buffer into the concentration of 0.01mg/ml.

Add 10 μL catalase or catalase nanocapsule test solution into the hydrogen peroxide buffer and mix them well by turning the well upside down. Then put the well into the UV/vis spectrophotometer to test the kinetic line of the reaction at 240nm. Based on the enzymatic assay of catalase (EC 1.11.1.6) from Sigma-aldrich, the time required for the $A_{240nm}$to decrease from 0.45 to 0.40 absorbance units was recorder to calculate the comparative activity.

2.3.4 Nanocapsule stability assay

SOD and nSOD were incubated with pepsin at 37°C in 10mM HCl solution for designed time points. 180 μl 10mM HCl, 10 μl pepsin solution (0.01-0.05 mg/ml) in10mM HCl and 10 μl SOD samples (1mg/ml) were added together. The activities of SOD samples were tested after the designed incubation time.

Catalase and nCatalase were incubated with trypsin at protein concentration of 0.5mg/ml in 50mm Tris-HCl(0.1% Ca^{2+}), pH=8.0 buffer at 37°C for several time points. Molar ratio of protein/trypsin=10:1. The activities of catalase were tested after designed incubation time.

2.3.5 *In vitro* cellular internalization

Cellular internalization studies were assessed via fluorescence microscopic technique. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine growth serum (FBS) and 1% penicillin/streptomycin. Cells (5000 cells/well, 96-well plate) were seeded the day

before adding the nanocapsules. After adding nanocapsules, keep incubation at 37 °C for 2 to 4 hrs, the cells were washed three times with PBS and visualized with a fluorescent microscope.

2.3.6 Cell viability assay

2.3.6.1 Under H_2O_2 environment

NIH 3T3 cells (5000 cells/well) were seeded on a 96-well plate 12 hrs before exposure to the catalase nanocapsules. nCatalase with different concentrations were incubated with the cells for 2-4 hrs, removed from the mixture, and incubated with fresh cDMEM media (0.001% H_2O_2) for 24 hrs. Then 0.1mg/ml resazurin[59] solution was diluted 10 times in completed DMEM media and was used to replace the media in each well and incubated for another 3 hrs. The plate was then placed inside a fluorescence plate reader. Excitation wavelength of 550nm and emission wavelength of 595nm was used.

2.3.6.2 Under superoxide radical abundant environment

NIH 3T3 cells (5000 cells/well) were seeded on a 96-well plate 12 hrs before exposure to nanocapsules. nCatalase and nSOD were incubated with the cells for 2-4 hrs, removed from the mixture, and incubated with different paraquat-concentrated fresh cDMEM media for 24 hrs. Then 0.1mg/ml resazurin solution was diluted 10 times in completed DMEM media and was used to replace the media in each well and incubated for another 3 hrs. The plate was then placed inside a fluorescence plate reader. Excitation wavelength of 550nm and emission wavelength of 595nm was used.

3 Results and discussion

The synthesis method for both nSOD and nCatalase are schematically showed in the Scheme 1 briefly. For nCatalase, catalase was modified with N-hydroxyl-succinimide ester first to generate acrylamide groups on the protein surface. Then it was mixed with 4-Dimethylaminoantipyrine(DMAAP), N,N'-methylenebisacryl amide(BIS), acrylamide(AAm), 2-(dimethylamino)ethyl methacrylate(DMAEMA) and the polymerization was initiated by APS/TEMED system. For nSOD, SOD was first modified with *N*-(3-aminopropyl)methacrylamide(APm), followed by polymerization using N,N'-methylenebisacryl amide(BIS), acrylamide(AAm), 2-(dimethylamino)ethyl methacrylate(DMAEMA).

To determine whether nanocapsules were formed successfully, agarose gel electrophoresis was used. Clear bands ran to the cathode can be observed which indicated the successful formation of positively-charged nanocapsules while native proteins ran to the anode because of their negative surface charge (fig. 1a,1b).

Sizes of nanocapules were also tested. For both nanocapsules, the sizes were between 10-30 nm (fig.2). It is expected that multiple proteins are encapsulated per nanocapsule. This size range is suitable for endocytosis. Surface charges of nanocapsules tested by zeta potential were suitable for intracellular delivery as well (table.1).

To test the activity of nSOD, we monitored the inhibition on the autoxidation of pyrogallol. SOD nanocapsule maintained 46.6% of the enzyme activity (fig. 1b).

Based on the time needed to break down certain amounts of hydrogen peroxide, the activity of catalase and nCatalase were tested. 83.6% of activity was maintained after encapsulation (fig. 1b).

Protease stability is always very important since proteins are always vulnerable to lot of proteases inside cells. Protease stabilities were also tested on both nSOD and nCatalase. Higher stability was showed in nCatalase(fig.3a). Better stability in nSOD was also obtained (fig.3b). The nanocapule shell showed great protection for enzymes against proteases.

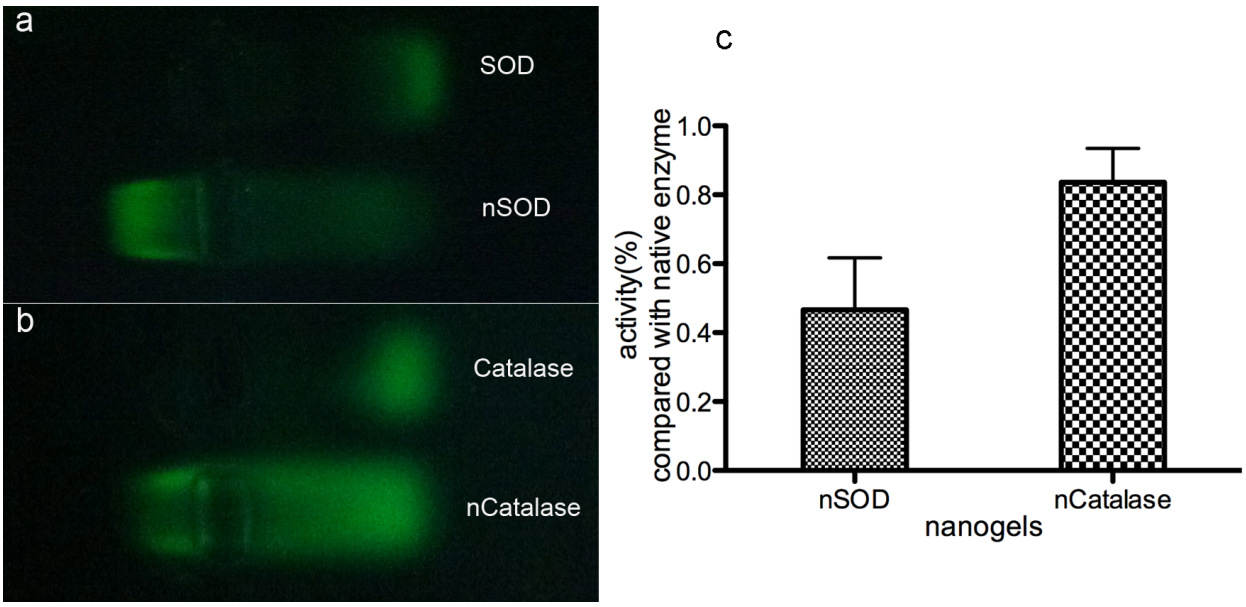


Figure 1. Nanocapsules formation and activities. (a) 0.8% agarose gel picture shows the formation of nSOD. (b) 0.8% agarose gel picture shows the formation of nCatalase. (c)Compared with the native enzymes at the same concentration, SOD nangels and Catalase nanocapsules maintains 46.6% and 83.6% of the activities, respectively.

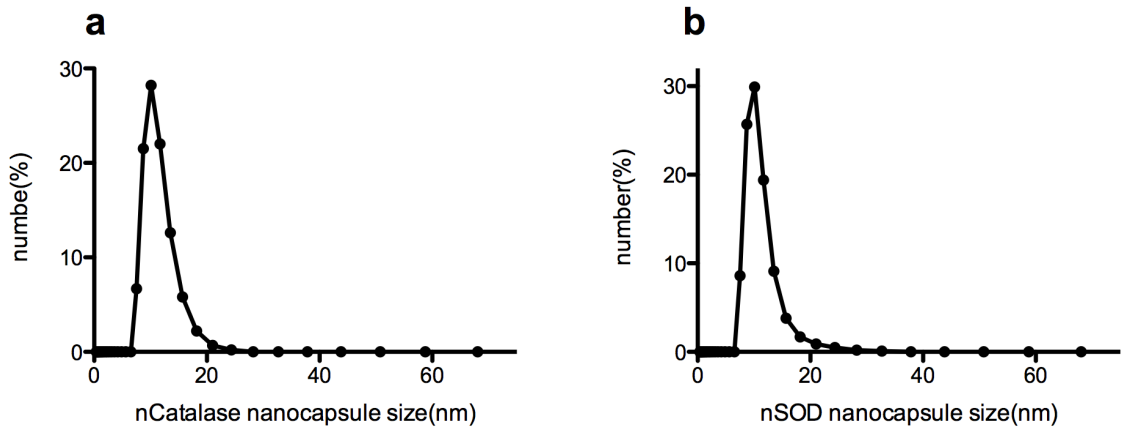


Figure 2. Size. (a)(b) both show that the size of nanocapsules are between 10-30 nm.

Samples	Zeta-potentials (pH=7.0)
Native SOD	- 4.69 mV
nSOD	+6.36 mV
Native Catalase	- 16.7 mV
nCatalase	-0.09 mV

Table 1. Zeta-potential of SOD, nSOD, catalase, nCatalase

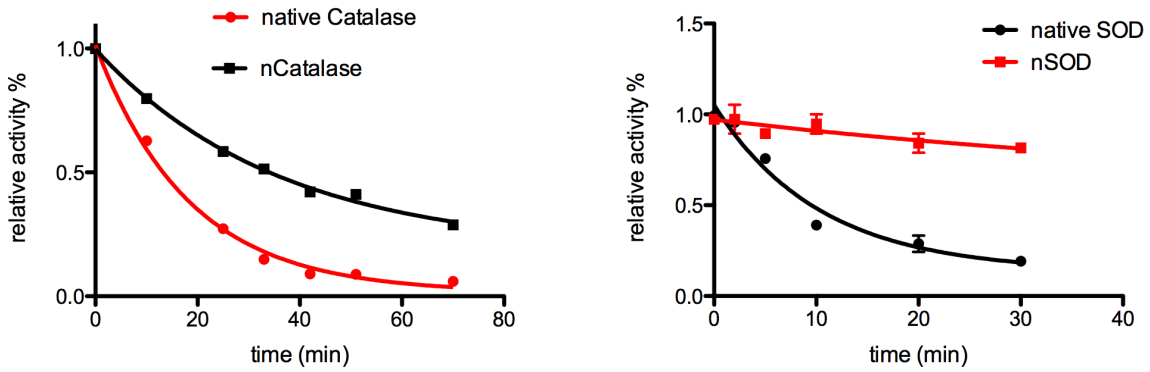


Figure 3. Nanocapules' protease stability. (a) Activity comparison of catalase nanocaosules and native catalase under trypsin incubation. (b) Activity comparison of SOD nanocapsules and native superoxide dismutase under pepsin treatment.

3.1 Cellular uptake of nanocapsules

Cellular uptake was studied using 400nM nSOD to incubate NIH3T3 cells. NIH3T3 cells can intake nSOD after 2 hrs incubation under 37 °C (fig.4 a). Clear fluorescence particles were observed which indicates the successful intracellular delivery.

The co-uptake of nSOD and n Catalase was also tested. nSOD was labeled with Rodamine while nCatalase was labeled with FITC. The incubation concentration in media was 5.96μM for nSOD and

0.153μM for nCatalase, respectively. Clear colocalization was observed (fig. 4b). This also gives a sound foundation for the synergy system.

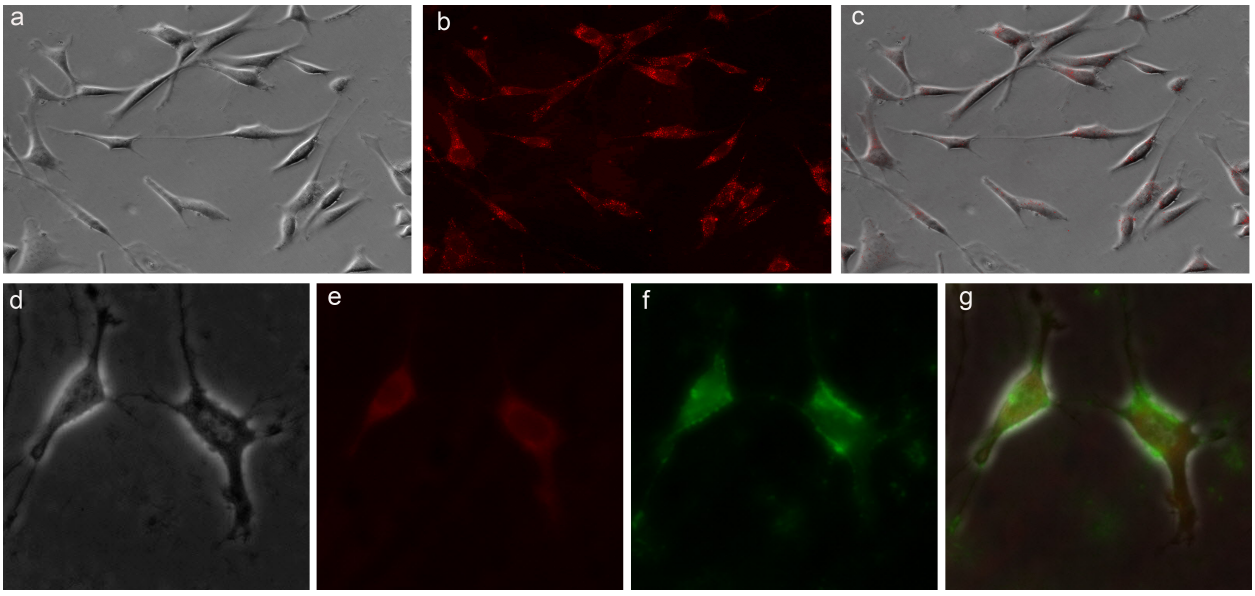


Figure 4. Cellular uptake images. (a)(b)(c) Fluorescent image shows uptake of nSOD into NIH 3T3 cells after incubation with nSOD for 2 hrs. (d)(e)(f)(g) Fluorescent image shows uptake of nSOD(labeled with Rodamin) and nCatalase (labeled with FITC) into NIH 3T3 cells after co-incubation with nSOD and nCatalase for 2 hrs

3.2 Increased cell viability is observed following the synergy incubation of nSOD and nCatalase

In order to test the effectiveness of nCatalase inside cells, NIH3T3 cells were first incubated 2 hrs with different volume of nCatalase (5μM) for 2 hrs. Later, after extensively washes using PBS buffer, completed DMEM media with 0.001% hydrogen peroxide was added into the 96-well plate for another incubation of 12 hrs. Later, media was changed into media containing resazurin (0.01mg/ml) and cell viability was tested via fluorescence. The cell viability of pre-nCatalase treated samples were significantly higher by more than 30 fold than those without pre-nCatalase incubation(fig. 5a). The activity of encapsulated catalase was thus confirmed inside cells. What's more, when without hydrogen peroxide and

with the increasing concentration of nCatalase incubation (from 50 to 200nM as final concentration), the cell viability was very steady and low cytotoxicity of nCatalase was observed (fig. 5a).

In order to optimize the synergy effect of nSOD and nCatalase, many different ratios of nSOD/nCatalase were tried using final nSOD concentration at 42.5 nM as standard. Paraquat, which has been long known for its quick autoxidation, was used at 0.4mmol/L to generate free hydrogen radicals. Compared with untreated control cell samples, when molar ratio of nSOD/nCatalase=5, the cell viability increased most (fig. 5b). However, when the ratio of nSOD/nCatalase was adjusted to 2.5, cell viability decreased a lot due to cytotoxicity. From fig.5b, nSOD itself showed no significant cell viability difference with untreated cells. After combined with nCatalase, at the molar ratio of nSOD/nCatalase=5, the synergy system increased the cell viability by 52.2%(fig. 5b). This molar ratio adjustment could also be used for other synergy enzyme nanocapules system to find the best ratio combination. After maintaining certain enzyme nanocapsules activity, by testing different ratios, the optimized ratios could be found easily.

Under final concentraions of nCatalase (40nM) and nSOD (200nM), nSOD/nCatalase=5, with the increase of concentrations of paraquat from 0.4mmol/L to 1.2mmol/L, the effect of synergy system gets more and more significant. At paraquat concentration of 1.2mmol/L, the synergy system obtained 2.65 fold of cell viability compared with untreated cells while nSOD and nCatalase only get 1.38 fold and 1.33 fold, respectively. From fig.5c, clear increasing trend of cell viability can be observed with the increasing toxicity when comparing synergy system with nCatalase or nSOD only system. The synergy system improves the protein functions by combining different nanocapsules together and boosts the cell viability.

Under paraquat concentration of 0.8mmol/L, when compared with control cells without nanocapsule treatement, the synergy nanocapsules system maintained 3.28 folds of cell viability while 1.25 folds for nCatalase only system and 1.19 folds for nSOD only system (fig. 5d). With the same dosage used separately, single enzyme system can improve but only around 20% of cell viability. With less amount of

nanocapsules at an optimized ratio and concentration, significant cell viability increase can be seen. This synergy system can be economical beneficial. It can also be seen clearly that under non-toxic environment, synergy nanocapsule system had 21.9% of toxicity compared with samples without nanocapsule treatment (fig.5d). However, under radical-toxic environment, the functions of nanocapsules surpassed the negative effect and achieve great cell viability.

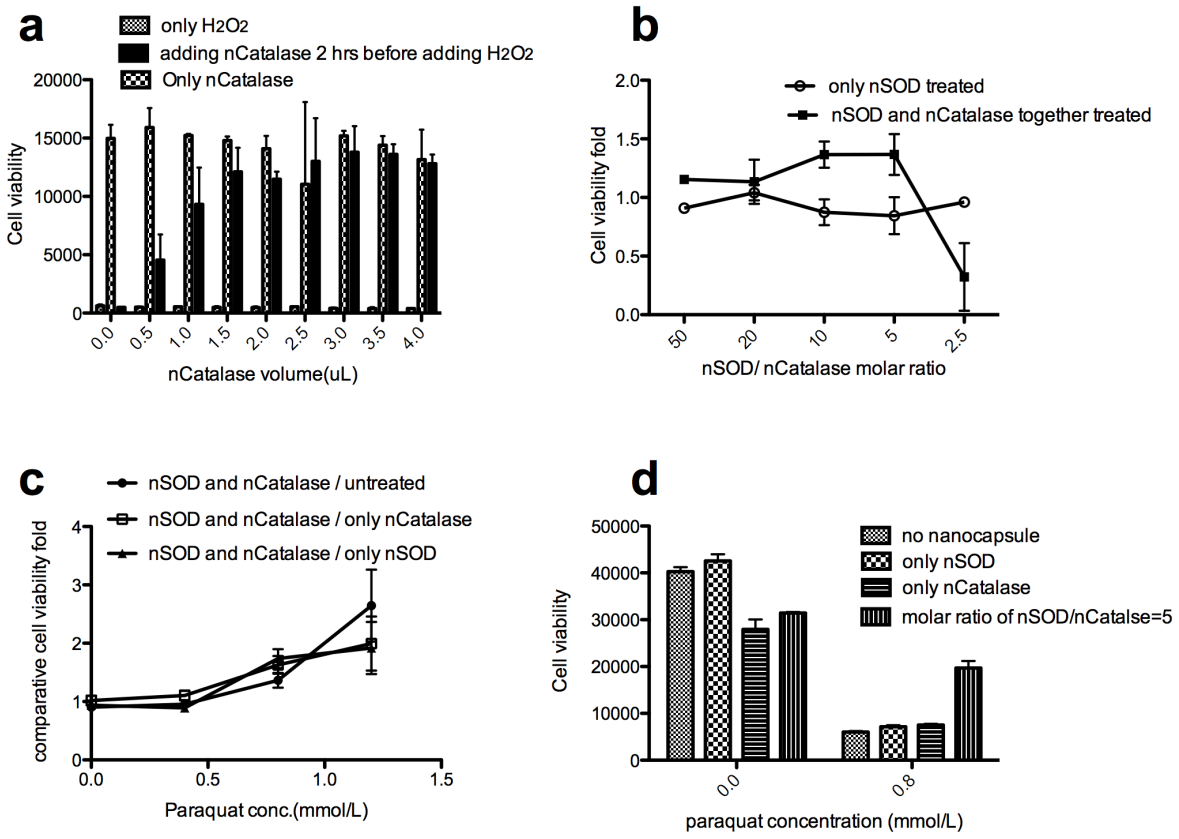


Figure 5. Cell viability test of bi-enzyme synergy system. (a) Cell viability of none-incubated, nanocapsule-incubated and only nanocapsule-incubated cells under different volume of nCatalase (5μM) for 2 hrs. (b) Cell viability fold change under different nSOD/ nCatalase ratio. The standard is untreated control cell samples. (c) Comparative cell viability fold under nSOD/nCatalase=5. (d) Cell viability when nSOD is 400nM and nCatalase is 80nM.

4 Conclusion

In conclusion, superoxide dismutase and catalase can be encapsulated inside positively charged polymer shell in situ polymerization. The diameter and charge of nanocapsules were suitable for intracellular delivery. The average diameter is from 10 to 30 nm. The intracellular deliveries were observed clearly via fluorescence microscope. Under radical-abundant environment, higher cell viability was observed when cells were incubated with both nanocapsules at the certain ratio of nSOD/nCatalase=5 compared with only nSOD or nCatalase. We believe that the use of this synergy delivery providing a sound progress for eliminating ROS *in vitro* via synergy protein therapy. What's more, this bi-enzyme nanocapsule system can also be seen as solid foundation for multi-enzyme nanocapsule system to achieve other complex therapeutic applications.

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