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Stable self-assembled nanostructured hen egg white lysozyme exhibits strong anti-proliferative activity against breast cancer cells

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ABSTRACT

Chemotherapy side effects have long been a matter of great concern. Here we describe a structurally stable self-assembled nanostructured lysozyme (snLYZ) synthesized using a simple desolvation technique that exhibited anticancer activity, as well as excellent hemocompatibility. Field emission scanning electron microscopy; atomic force microscopy and dynamic particle size analyzer were used for analyzing the synthesized snLYZ. The analysis revealed spherical shape with an average size of 300 nm. Circular dichroism and tryptophan fluorescence spectroscopic analysis revealed its gross change in secondary as well as the tertiary level of the structure. snLYZ also demonstrated excellent structural as well as the functional stability of LYZ in a wide range of pH and temperature with a fair level of protection against proteinase K digestion. When applied to MCF-7 breast cancer cells, it exhibited approximately 95% cell death within 24 h, involving a reactive oxygen species (ROS) based mechanism, and showed excellent hemocompatibility. Fluorescence microscopy imaging revealed distinct cellular internalization of snLYZ and the formation of cytoplasmic granules, which initiated a cell-killing process through membrane damage. In order to mimic targeted therapy, we tagged folic acid with snLYZ, which further enhanced cytotoxicity against MCF-7 cells. Therefore, this is the first report of its kind where we demonstrated the preparation of a highly stable self-assembled nanostructured lysozyme with a strong anti-proliferative activity against breast cancer cells.

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1. Introduction

Proteins are highly sensitive to external stress because of their intimate link between 3-D structure and biological function [1–3]. Proteins also possess unique properties to form self-assembled nanostructures. They form disordered as well as ordered aggregates in cells. In many instances, such accumulation is the cause of number of lethal diseases like Amyloidosis, Alzheimer's, Parkinson's and Prion disease [4–7]. However, protein structure and their self-assembly process are externally driven and has found many applications in biology and medicine [8–15]. Nanoparticles, developed from natural biopolymers are usually biodegradable, easy to metabolize, and easily surface modifiable, which may facilitate efficient attachment of drugs [16]. Such nanoparticles have been reported to develop from various proteins including bovine and human serum albumin, zein and gliadin [16].

Recent reports cite special preparation of small proteins such as bovine and human α-lactalbumin, to be lethal against a number of tumor cell types. Specific conformational state of proteins was also reported to form conjugates with oleic acid known as BAMLET (Bovine α-lactalbumin made lethal to tumor cells) or HAMLET (Human α-lactalbumin made lethal to tumor cells). These conjugates also induced tumor cell death [17,18]. Therefore, specific structural state of such proteins may have a therapeutic potential.

Here we report the use of hen egg white lysozyme (LYZ) for the preparation of self-assembled nanostructured lysozyme (snLYZ). Breast cancer cells were treated with snLYZ for the evaluation of its anticancer activity. The molecular mass of LYZ is 14.4 kDa and composed of 129 amino acids [19]. It is active site contains a deep crevice, which divides the protein into two separate domains linked by α helix. The first domain (residues 40–85) consists mostly of β-sheet structure, whereas the second domain (residues 89–99) is more helical in nature [20].

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Recently, lysozyme extracted from a marine bacterium has demonstrated to inhibit angiogenesis and tumor growth in mice [21]. Lysozyme has also shown tumorigenic activity in several other studies [22].

The idea behind using LYZ to prepare self-assembled nanostructure for exploring its potential in breast cancer therapy developed from its earlier reports of antitumorigenic and anticancer potential. Caselli and Schumacher first reported the antitumorigenic activity of native lysozyme (nmLYZ) [20]. Two remarkable studies performed by Babudieri-Callerio and Callerio [23,24] demonstrated the effect of LYZ on H.S1 (mesenchymal origin), Hep2 (epithelial nature) strains and HeLa cells. The LYZ treatment produced granular irregular structures in the cytoplasm. Sava et al. in 1989 reviewed the anticancer potential of exogenous native LYZ exhaustively, where he reviewed various models and mechanism of action of LYZ-mediated cancer cell death [25]. Lysozyme was used earlier for antitumor activity in many in vivo model systems of animal tumors [26–28]. However, after 1989, except for the anti-proliferative effect of recombinant human lysozyme on gastric cancer cells [29], no significant progress was made toward cancer therapeutic approach using LYZ.

Using a simple desolvation technique we have prepared self-assembled nanostructured lysozyme (snLYZ) and evaluated its possible anticancer activity. We thoroughly characterized snLYZ concerning its structure, function and stability under various stress. After a series of experimental studies, we established its strong anti-proliferative activity against breast cancer cells.

2. Materials and methods

2.1. Materials

N-acetyl-cysteine (NAC), Glutaraldehyde (25%), Tamoxifen citrate and Whatman filter paper were purchased from Sigma-Aldrich, India. Lysozyme, Bovine serum albumin, Folic acid (FA), MTT assay kit, Ethanol, DMEM, fetal bovine serum (FBS), antibiotics, DAPI, acridine orange, Fluorescein diacetate, phenylmethanesulfonyl fluoride (PMSF), Proteinase K, sodium cacodylate, sodium phosphate buffer, 1-anilinonapthalene-8-sulfonic acid and disodium EDTA were purchased from HiMedia India Pvt. Ltd. Milli Q water was used in all the experiments. T-25 flasks, 96 well plates and all other plastic wares were purchased from Tarsons, India Pvt. Ltd. 3T3 (murine fibroblast cell line), HaCaT (human keratinocyte cell line), A549 (human lung adenocarcinoma epithelial cell line) and MCF-7 (breast cancer cell line) cells were procured from NCCS, Pune, India. Triton-X was purchased from Calbiochem, India. Human blood (B +ve) was freshly obtained from the donor before the experiments. All glassware used in the study purchased from Borosil, India. All other reagents were of analytical grade.

3. Methods

3.1. Preparation of self-assembled nanostructured lysozyme (snLYZ) and its conjugates

Lysozyme (10 mg) was dissolved in 2 ml of Milli-Q water. The contents were stirred for 15 min at 500 rpm. During stirring, 8 ml of ethanol was added dropwise (1 ml/min) using an insulin syringe. After addition of ethanol, the color of the solution turns turbid white. Immediately glutaraldehyde (cross-linker) was added to achieve a final concentration of 0.1%. The contents were allowed to stir for 8 h at 500 rpm followed by 5 cycles of centrifugation (25,000 × g, 30 min, 40 °C). After centrifugation, the pellet was dispersed in 3 ml of Milli-Q water. The frequent washing and five cycles of centrifugation were carried out, in order to remove the residual glutaraldehyde from the formulation.

During the process of optimization, snLYZ was prepared using various concentrations of glutaraldehyde (GTD) such as 0.1%, 0.3%, 1% and 3%. The activity assay of LYZ (see Section 3.3.3), field emission scanning electron microscopy (FESEM) imaging (see Section 3.2.1), circular dichroism (CD) and tryptophan (trp) fluorescence spectroscopy (see Section 3.2.4) were performed for the optimization of GTD concentration. Although various concentration of GTD were used in the preparation of snLYZ, the optimized GTD concentration, i.e., 0.1% was used in the preparation of snLYZ and subsequent experiments.

We prepared the snLYZ-folic acid (snLYZ-FA) conjugates with the aim of increasing the specificity of snLYZ for cancer cells. For the preparation of snLYZ-FA conjugate, the prepared snLYZ was suspended in 5 ml of Milli-Q water, followed by the addition of 25 μl of 2 mg/ml stock solution of FA. The contents were stirred for 8 h at 500 rpm in the presence of 0.1% GTD. The rest of the procedure remains same as mentioned above.

The schematic representation of the whole synthesis protocol was shown in Fig. 1.

The same procedure was also followed for the preparation of self-assembled nanostructured bovine serum albumin (snBSA), a 66 kDa monomeric protein, for use as a control of snLYZ.

3.2. Characterization of prepared snLYZ

3.2.1. Field emission scanning electron microscopy (FESEM)

For capturing high-resolution images of various samples, FESEM study was performed. A volume of 10 μl of snLYZ (2 mg/ml) was spread on a glass slide and allowed to dry in a desiccator. The glass slides with the samples were fixed on a carbon tape attached to the aluminum stub. The aluminum stub was placed in a gold sputtering unit for 30 s. The samples were subsequently placed in the FESEM (NOVA NANO SEM 450) sample chamber and images were captured at a voltage of 5 kV.

3.2.2. Atomic force microscopy (AFM)

AFM imaging and measurements were performed using Bioscope Catalyst AFM (Bruker Corporation, Billerica, MA) with silicon probes. A volume of 10 μl of snLYZ (2 mg/ml) was placed on a freshly peeled mica surface followed by drying under a continuous flow of nitrogen gas. The mica discs were gently washed with 200 μl of Milli-Q water. The mica discs were allowed to dry in a desiccator overnight. The standard tapping mode was used for capturing images of different samples at room temperature in the presence of air. The nominal spring constant of the cantilever used was 20–80 N/m. A standard scan rate of 0.5 Hz with 512 samples per line was used for imaging the samples. AFM image of the snLYZ sample was captured the next day.

3.2.3. Dynamic light scattering (DLS) particle size analysis and zeta-potential analysis

The average particle size analysis and ζ-potential measurement of the aqueous suspensions of snLYZ were performed on a Malvern Zetasizer Nano-ZS DLS analyzer. A sample volume of 10 μl of snLYZ (2 mg/ml) was taken and diluted with 1990 μl of deionized water and applied to DLS for size analysis.

3.2.4. Circular dichroism (CD) and tryptophan fluorescence measurement

CD spectra of nmLYZ and snLYZ (200 μg/ml) was recorded using a JASCO J-815 CD polarimeter. Sodium cacodylate (20 mM) at pH 7.4 was used as a buffer for measuring the CD spectra. All the measurements were carried out at physiological temperature (37 °C) and a constant flow of N_2 gas. Each spectrum represented the

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