Biochemistry and Biophysics Reports 7 (2016) 225–229

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Glutathione disulfide liposomes – A research tool for the study of glutathione disulfide associated functions and dysfunctions

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ARTICLE INFO

Article history:
Received 21 February 2016
Received in revised form
18 June 2016
Accepted 20 June 2016
Available online 28 June 2016

Keywords:
Glutathione disulfide
Liposomes
Glutathionylation
Oxidative stress
Thiols

ABSTRACT

Glutathione disulfide (GSSG) is the oxidized form of glutathione (GSH). GSH is a tripeptide present in the biological system in mM concentration and is the major antioxidant in the body. An increase in GSSG reflects an increase in intracellular oxidative stress and is associated with disease sates. The increase has also been demonstrated to lead to an increase in protein *S*-glutathionylation that can affect the structure and function of proteins. Protein *S*-glutathionylation serves as a regulatory mechanism during cellular oxidative stress. Though GSSG is commercially available, its roles in various GSSG-associated normal/abnormal physiological functions have not been fully delineated due to the reason that GSSG is not cell membrane permeable and a lack of method to specifically increase GSSG in cells. We have developed cationic liposomes that can effectively deliver GSSG into cells. Various concentrations of GSSG liposomes can be conveniently prepared. At 1 mg/mL, the GSSG liposomes effectively increased intracellular GSSG by 27.1 $\pm$ 6.9 folds (n=3) in 4 h and led to a significant increase in protein *S*-glutathionylation confirming that the increased GSSG is functionally effective. The Trypan blue assay demonstrated that GSSG liposomes were not cytotoxic; the cell viability was greater than 95% after cells were treated with the GSSG liposomes for 4 h. A stability study showed that the dry form of the GSSG liposomes were stable for at least 70 days when stored at $-80\,^{\circ}C$. Our data demonstrate that the GSSG liposomes can be a valuable tool in studying GSSG-associated physiological/pathological functions.

1. Introduction

Glutathione disulfide (GSSG) is the oxidized form of glutathione (GSH). GSH is an endogenous three amino acid peptide present in mM concentration in cells and serves as the major antioxidant in the biological system [1]. GSH protects the biological system from oxidizing factors such as reactive oxygen species (ROS) or reactive nitrogen species (RNS) through terminating them while GSH itself is oxidized to GSSG. GSSG is then reduced back to GSH by glutathione reductase (GR) to maintain thiol redox homeostasis (Fig. 1). Under normal conditions, the biological system maintains a high ratio of GSH:GSSG (> 100:1) through effective reduction of GSSG back to GSH. An increase in GSSG is considered as an increase in cellular oxidative stress [2].

An increase in GSSG has also been implicated in various diseases such as neurodegenerative diseases [3] and cystic fibrosis [4]. Further, an increase in GSSG was demonstrated to increase protein *S*-glutathionylation [5]. Protein *S*-glutathionylation is involved in oxidative stress and structural and functional modification of proteins. Protein *S*-glutathionylation also serves as a cellular regulatory mechanism like protein phosphorylation [5]. A study of the effects of GSSG changes on GSSG-associated physiological/pathological states and protein S-glutathionylation remains challenging due to a lack of a research tool to specifically increase intracellular GSSG levels since GSSG is a cell membrane impermeable molecule. Current methods to increase intracellular GSSG levels mainly include a microinjection approach [6] and use of GSSG methyl ester [7]; the latter is expected to be hydrolyzed to yield GSSG intracellularly. The microinjection approach requires an expertize and is not applicable for most *in vitro* and *in vivo* studies. The obvious drawback of GSSG methyl ester is that the rate of hydrolysis by esterases may not be the same in different cells. Also, GSSG methyl ester is not suitable for *in vivo* study since it will be quickly hydrolyzed in plasma before it can reach the targeted site.

Abbreviations: GSSG, glutathione disulfide; GSH, glutathione; DDAB, dimethyldioctadecylammonium bromide; PBS, phosphate buffered saline; FBS, fetal bovine serum; BBB, blood-brain barrier

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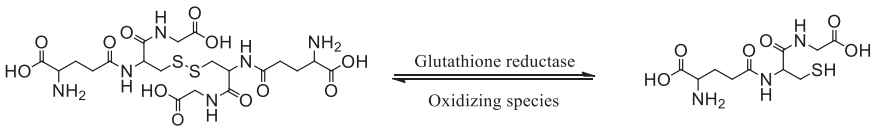
http://dx.doi.org/10.1016/j.bbrep.2016.06.017



GSSG GSH

Fig. 1. Chemical structure of GSSG and its relationship with intracellular antioxidant GSH.

We reported early a method to increase intracellular GSSG through the use of a GR inhibitor [8]. However, the inhibitor was later found also to inhibit other disulfide reductases such as glutaredoxin [9] and thioredoxin reductase [10]. Therefore, the inhibitor is more appropriate as a tool to block all disulfide reduction to increase cellular thiol oxidative stress but not selective enough for the study of the impact caused by GSSG alone.

Here we would like to report a method that effectively delivers GSSG into cells through the use of cationic liposomes. The increase of GSSG led to a significant increase in cellular protein *S*-glutathionylation confirming that the delivered GSSG is functionally effective. This is the first method that delivers specifically GSSG, not in the other forms of GSSG such as GSSG methyl ester, into cells. The method will be valuable in studying the impact of GSSG on GSSG-associated cellular function/dysfunction and provide unambiguous information regarding the roles of GSSG in these function/dysfunction. Similarly, it will be valuable in studying protein *S*-glutathionylation-related function/dysfunction.

2. Material and methods

2.1. Materials

Lecithin (Phospholipon 90 G) was obtained as a gift from Lipoid (Ludwigshafen, Germany). Cholesterol was supplied by MP Biomedicals (Solon, Ohio), dimethyldioctadecylammonium bromide (DDAB) and GSSG were obtained from Applichem (Darmstadt, Germany). GSH was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Chloroform and LC/MS grade acetonitrile, water and formic acid were obtained from Fisher Scientific (Fair Lawn, NJ). 5-Sulfosalicylic acid was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). RPMI 1640 growth medium, penicillin/streptomycin, phosphate buffered saline (PBS), and trypsin were obtained from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). NCI-H226 cells (human non-small cell lung carcinoma) were obtained from the National Cancer Institute.

2.2. Preparation of GSSG cationic liposomes

The GSSG liposomes were prepared using a dehydration/rehydration method with minor modification [11]. Briefly, Lecithin (70 mg), cholesterol (10 mg) and DDAB (20 mg) were dissolved in chloroform (10 mL), and evaporated to dryness to form a thin and homogeneous lipid film by rotation in a 250 mL round bottom flask under a reduced pressure at 40 °C. The lipid film was then hydrated with a GSSG PBS loading solution (GSSG dissolved in PBS) (10 mL). Size reduction of the resulting suspension was performed by sonication in a bath sonicator at 40 °C (4 min sonication with 1 min break) for 20 min. After sonication the resulting liposomal suspension was distributed in aliquots of 1 mL each and frozen at −80 °C for 12 h, freeze-dried, and stored at −80 °C for later use.

To use the stored freeze-dried GSSG liposomes, the liposomes in the vial were reconstituted in water (1 mL) (rehydration), vortex mixed for 1 min, and allowed to stand at 40 °C for 10 min, then at room temperature for additional 20 min. The reconstituted liposomes were loaded to a Sephadex column (PD10 Columns, GE Health care, Little Chalfont, UK) and centrifuged to yield the GSSG liposomes. GSSG liposomes were prepared with equal amount of GSSG and lipid in the liposome formulation: GSSG liposomes at 1 mg/mL mean the liposome formulation contained 1 mg GSSG and 1 mg lipid.

2.3. Determination of particle size and zeta potential

The above rehydrated liposomes were further subjected to a dilution of 1:100 with deionized water and then examined for particle size and zeta potential by photon correlation spectroscopy and Doppler anemometry using Malvern Zeta One Zeta Potential/Particle Sizer.

2.4. Determination of GSSG loading

The liposome samples prepared above were diluted further using 3% sulfosalicylic acid (1:10 dilution) and sonicated for 1 h. This sonicated sample was further diluted in 0.1% trifluoroacetic acid (1:100 dilution). The resulting solution was centrifuged at 14,000 rpm for 3 min before analysis using LC/MS/MS. LC/MS/MS quantification of GSSG employed an Agilent Eclipse XDB-C18 column (1.0 × 150 mm, 3.5 μm) equipped with an Agilent ZORBAX Eclipse XDB guard column (1.0 × 17 mm, 5 μm). The mobile phase was composed of 0.1% formic acid and acetonitrile at a ratio of 92/8 (v/v). The flow rate was set at 70 μL/min. In MS/MS detection, the electrospray ion source was operated in the positive ionization mode with the following optimized parameters: sheath gas 85 arb, spray voltage 4.5 kV, capillary temperature 350 °C, capillary voltage 3 V and tube lens offset −60 V. Selected reaction monitoring (SRM) was performed to monitor the mass transition of 613→484 (GSSG). Under these conditions, the retention time of GSSG was 3.0 min. The injection volume was 1 μL. GSSG was quantified based on a GSSG standard curve constructed right after the GSSG sample analysis.

2.5. Stability of the stored freeze-dried GSSG liposomes

The GSSG liposomes prepared above were distributed 1 mL each in a 1 mL centrifuge vial, frozen at −80 °C, freeze-dried, and then stored at −80 °C. Periodically, vials containing the freeze-dried GSSG liposomes were reconstituted with water (1 mL), passed through a Sephadex column to remove nonencapsulated GSSG, and analyzed for the encapsulated liposomal GSSG by LC/MS/MS as described above. The stability of the stored freeze-dried GSSG liposomes was determined over a period of 70 days.

2.6. Cellular uptake of GSSG liposomes in NCI-H226 cells

Exponentially growing NCI-H226 cells (4×10^6) were placed in a 75 cm^2 flask in RPMI 1640 growth medium containing 10% FBS and 1% penicillin/streptomycin in a 5% CO_2 incubator at 37 °C for 12 h for attachment before treated with GSSG liposomes[1] (1 mg/mL)[2] in a 5% CO_2 incubator at 37 °C. At the end of the

[1] GSSG liposomes for cell culture use were prepared by reconstituting the stored freeze-dried liposomes with sterile water instead of deionized water.

[2] 1 mg of GSSG and 1 mg of lipid.