Research Article

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Acrylamide-encapsulated glucose oxidase inhibits breast cancer cell viability

[Akrilamid-enkapsüle glukoz oksidaz meme kanseri hücre canlılığını inhibe etmektedir]

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Abstract

Objectives: Cancer cells modulate metabolic pathways to ensure continuity of energy, macromolecules and redox-homeostasis. Although these vulnerabilities are often targeted individually, targeting all with an enzyme may prove a novel approach. However, therapeutic enzymes are prone to proteolytic degradation and neutralizing antibodies leading to a reduced half-life and effectiveness. We hypothesized that glucose oxidase (GOX) enzyme that catalyzes oxidation of glucose and production of hydrogen peroxide, may hit all these targets by depleting glucose; crippling anabolic pathways and producing reactive oxygen species (ROS); unbalancing redox homeostasis.

Methods: We encapsulated GOX in an acrylamide layer and then performed activity assays in denaturizing settings to determine protection provided by encapsulation. Afterwards, we tested the effects of encapsulated (enGOX) and free (fGOX) enzyme on MCF-7 breast cancer cells.

Results: GOX preserved 70% of its activity following encapsulation. When fGOX and enGOX treated with guanidinium chloride, fGOX lost approximately 72% of its activity, while enGOX only lost 30%. Both forms demonstrated remarkable resilience against degradation by proteinase K and inhibited viability of MCF-7 cells in an activity-dependent manner.

Conclusions: Encapsulation provided protection to GOX against denaturation without reducing its activity, which would prolong half-life of the enzyme when administered intravenously.

Keywords: biocompatibility; cancer therapy; glucose oxidase; MCF-7; single enzyme nanoparticle; starving-like therapy; therapeutic enzyme.

Özet

Giriş: Kanser hücreleri, enerji devamlılığı ile makromolekül üretimini ve redoks homeostazını sağlamak amacıyla metabolik yolakları modüle ederler. Bu hassas noktalar ilaç geliştirme çalışmalarında ayrı ayrı hedeflense de, tümünü bir enzim aracılığıyla aynı anda hedeflemek yeni bir yaklaşım niteliği taşımaktadır. Bununla birlikte, terapötik enzimler ilaç olarak uygulandığında etkinliğinde azalmaya yol açan proteolitik degradasyon ve nötralize-edici antikorlara açık durumdadır.

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Amaç: Glukozun oksidasyonunu ve hidrojen peroksit üretimini katalizleyen enzim olan glukoz oksidazın (GOX), mikroçevredeki glukozu tüketerek sentez ve enerji üretim yolaklarını engelleme ve redoks homeostazının dengesini bozacak reaktif oksijen türleri açığa çıkarma yollarıyla tüm bu hedefleri vurabileceği hipotezini kurduk.

Materyal ve Metotlar: Enzimi akrilamid tabaka içine enkapsüle ettik ve enkapsülasyon tarafından sağlanan korumanın düzeyini, denatüre edici koşullarda aktivite denemeleri ile belirledik. Son olarak, hem serbest (fGOX) hem de enkapsüle GOX'un (enGOX) MCF-7 meme kanseri hücreleri üzerindeki etkilerini belirledik.

Sonuçlar: Enkapsülasyon sonrası GOX, aktivitesinin % 70'ten fazlasını korumuştur. GndCl ile muamele edildiğinde, fGOX aktivitesinin %72'sini kaybederken enGOX aktivitesinin yalnızca %30'unu kaybetmiştir. Her iki form da, proteinaz K ile parçalanmaya karşı kayda değer bir direnç sergilemiş ve MCF-7 meme kanseri hücrelerinin canlılığını aktiviteye bağımlı şekilde inhibe etmiştir.

Tartışma: Enkapsülasyon GOX'un aktivitesini azaltmadan denatürasyona karşı koruma sağlamıştır. Bu sayede, intravenöz olarak uygulanması durumunda enzimin yarı ömrü uzatılmıştır.

Anahtar kelimeler: biyouyumluluk; Glukoz oksidaz; kanser tedavisi; MCF-7; starvasyon terapisi; tek enzim nanopartikülü; terapötik enzim.

Introduction

Despite the fact that cancer cells are highly heterogeneous, they still share some common hallmarks that can be targeted for therapeutic purposes, such as sustaining proliferative signaling, resisting cell death, enabling replicative immortality and deregulating cellular energetics [1]. The last hallmark represents a modulation of cellular metabolic pathways to ensure efficient ATP production, continuous macromolecule synthesis and balanced redox status [2].

Metabolic alterations in glucose metabolism are important targets for cancer treatment and there are many studies showing that inhibiting aerobic glycolysis suppresses tumor growth [3]. One of the possible strategies to target cellular metabolic pathways is using active enzymes instead of small molecules as therapeutic agents. This strategy has already been tested in cancer as L-asparaginase, an enzyme degrading asparagine, is still being used to treat acute lymphoblastic leukemia decades after it was first developed [4–6]. However, since enzymes are essentially protein-based molecules, they are prone to changes in pH, shear force, proteolytic degradation and neutralizing antibodies. Chemical modification of the enzymes is commonly used to enhance protein half-life and one such example is PEGylation of L-asparaginase to obtain PEG-asparaginase which was approved by the Food and Drug Administration (FDA) as Oncaspar® [6]. Asparaginase has been known to kill leukemic cells by depleting their asparagine pools and is especially useful as a treatment option for acute lymphocytic leukemia (ALL) [7].

In this study, we have decided to focus on an enzyme that targets glucose, the main energy and carbon source of cancer cells. Glucose oxidase (GOX) is naturally produced in fungus *Aspergillus niger* and catalyzes the conversion of glucose to hydrogen peroxide and D-glucono-δ-lactone [8]. We hypothesized that GOX would inhibit cell viability due to its enzymatic activity which catalyzes depletion of glucose and production of reactive oxygen species (ROS). We also hypothesized that encapsulation of the enzyme protects against half-life reducing effects. We used MCF-7 breast cancer cells as an *in vitro* model and treated the cells with free and encapsulated GOX to test the effects of the enzyme.

Materials and methods

Glucose oxidase encapsulation by acryloylation and in situ polymerization – To encapsulate GOX enzyme molecules, we performed a two-step method previously described by Ming Yan [9] . The first step, acryloylation reaction, introduces vinyl groups to the surface of the enzyme. Briefly 20.6 µL of 50 mM GMA (Glycidyl methacrylate) (Sigma–Aldrich, MO, USA), 19.4 µL of 1 M DMSO (Dimethyl sulfoxide) (Sigma–Aldrich, MO, USA), 400 µL of 10 mg/mL GOX enzyme solution (*A. niger*, type II, EC number 1.1.3.4) (Sigma–Aldrich, MO, USA), and 560 µL of 50 mM pH 8.5 sodium carbonate buffer were added to a 2 mL tube. The mixture was incubated at 37 °C, 150 RPM for 2 h for the reaction to occur. The mixture was then desalted using a G-10 desalting column to remove GMA-DMSO residues and acryloylated enzyme was collected from the column using 0.1 M, pH 7.2 PBS buffer solution. We performed this reaction with increasing concentrations of GMA to optimize the encapsulation.

The second step of encapsulation process was polymerization. Acrylamide >99% (Sigma–Aldrich, MO, USA) and *N*,*N*-Methylenebisacrylamide 99% (Sigma–Aldrich, MO, USA) were added to 2 mL of 0.2 mg/mL acryloylated GOX solution. We used different concentrations of acrylamide and *N*,*N*′-Methylenebisacrylamide to optimize the particle size (Table 1). Radical polymerization was initiated by the addition of ammonium persulfate 98% (0.1 mg/mL) and *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) 99% (13.26 mM) (Sigma–Aldrich, MO, USA) to the 2 mL tubes. Reaction was carried out at 4 °C for 24 h with continuous stirring. After 24 h resulting nanoparticles containing encapsulated enzyme were desalted using a PD-10 column and activity of GOX was measured to confirm presence of the enzyme. Final nanoparticles containing the active enzyme were eluted from columns with 0.1 M, pH 7.2 PBS. Concentrations tested during encapsulation are summarized in Table 1.

Table 1: Concentration of enzyme, acrylamide and bisacrylamide used in polymerization.

Sample	Acryloylated enzyme, mg/mL	Acrylamide, mg/mL	*N,N′*–Methylenbisacrylamide, µg/mL
1	0.2	–	–
2	0.2	0.185	2
3	0.2	0.37	4
4	0.2	0.74	8
5	0.2	1.48	16
6	0.2	2.96	32

GOX activity assay – The activity of the enzyme was measured using a horseradish peroxidase (HRP) (type I, EC number 1.11.1.7) (Sigma–Aldrich, MO, USA) coupled colorimetric assay. The H_2O_2 produced by GOX is used by peroxidase to convert ABTS (2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) [Sigma–Aldrich, MO, USA]) to its oxidized form, which shows high absorbance at 420 nm. Oxidized ABTS absorbance is directly proportional to GOX activity. Absorbance at 420 nm was measured with Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific, MA, USA).

Denaturation and degradation of GOX – A denaturation experiment was performed using increasing concentrations of guanidinium chloride (GndCl), a chaotropic agent that disrupts the protein structure by affecting the non-covalent hydrogen bonds, to determine if encapsulation of GOX provided denaturation resistance. Samples of both types of enzyme (0.75 µM of encapsulated or free enzyme) were mixed with GndCl solutions of concentrations ranging from 0 to 8 M. These mixtures were then incubated in room temperature for one and a half hour. In order to determine if encapsulation protects GOX against proteolytic degradation, enzyme activity of fGOX and enGOX were measured after 2 h of proteinase K (enzyme:protein ratio 1:1) treatment at 37 °C with constant stirring.

Cell culture and determination of cell viability – In order to determine the effect of encapsulated GOX particles on the viability of MCF-7 breast cancer cells, crystal violet viability test was used. MCF-7 cells were incubated in complete cell culture medium, which was prepared using RPMI-1640 (Gibco/Thermo Fisher, MA, USA) with 10% fetal bovine serum (Biowest, Nuaillé, France), 1% L-glutamine (Gibco/Thermo Fisher, MA, USA) and 1% penicillin/streptomycin (Gibco/Thermo Fisher, MA, USA). Sterile PBS w/o Ca^{++} and Mg^{++} (GE Healthcare Life Sciences, PA, USA) was used to rinse and handle, and trypsin-EDTA (0.25%) sterile solution (Gibco/Thermo Fisher, MA, USA) was used to dissociate cells. Cells were incubated in humidified CO_2 incubator (Thermo, MA, USA) at 37 °C under 5% CO_2 pressure. During the assay, 10,000 cells were seeded in each well of a 96 well plate with 50 µL complete cell culture medium. Plate was incubated for 24 h at 37 °C, under 5% CO_2 pressure. Subsequently, GOX solutions were prepared in complete cell culture medium at activities ranging from 3×10^{-5} to 6×10^{-5} U and were added to the cells. 24 h more of incubation under the same conditions followed. After incubation cells were rinsed with sterile PBS. Viable cells were then stained with crystal violet (Sigma–Aldrich, MO, USA) solution prepared in 2% ethanol (Sigma–Aldrich, MO, USA) which enables permeation of the dye from the cell membrane. After that, cells were rinsed with PBS to remove excess dye and the stain was solubilized by 10% SDS (Sigma–Aldrich, MO, USA). Absorbance at 570 nm wavelength was measured using BioTek Synergy HTX (VT, USA). The experiment was designed to have four replicates (n=4) for each enzyme concentration. Viability percentages were calculated using absorbance values considering untreated cells as 100% viable controls. The same experiments were carried out using fGOX and enGOX.

Results

Encapsulation did not hinder GOX activity drastically. – GOX preserved more than 70% of its activity following its encapsulation by acryloylation and *in situ* polymerization in the highest tested concentrations of GMA (Figure 1A) and acrylamide (Figure 1B). Therefore, we decided to move on with these concentrations (1.03 mM GMA and 2.96 mg/mL acrylamide) in cell culture experiments.

Encapsulated GOX is more stable against denaturing agents. – When fGOX and enGOX were treated with guanidinium chloride (GndCl), the fGOX lost approximately 72% of its activity, while the enGOX only lost 30% (Figure 2A). Therefore, it is safe to assume that the encapsulation provided protection against denaturation. On the other hand, since the free form itself resists well against proteolytic degradation, encapsulation did not provide an extra protection from digestion. Both fGOX and enGOX demonstrated remarkable resilience against degradation by proteinase K digestion after 2 h of exposure (Figure 2B).

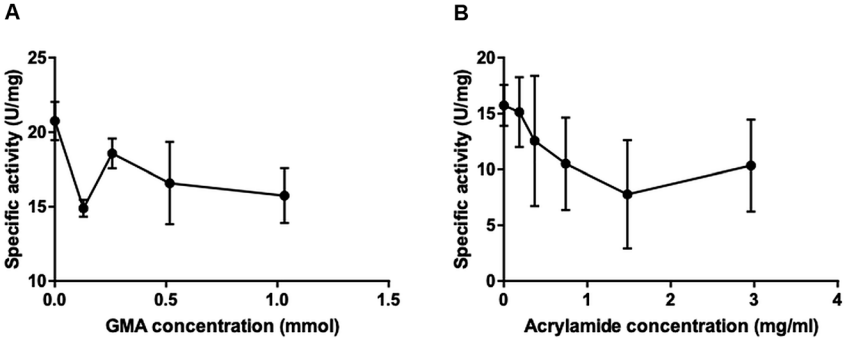


Figure 1: Effect of encapsulation on enzyme activity. Neither acryloylation nor polymerization affects enzyme activity to a great extent. (A); Activity of glucose oxidase after acryloylation (n=3, error bars = SD), and (B); after in situ polymerization with acrylamide (n=3, error bars = SD).

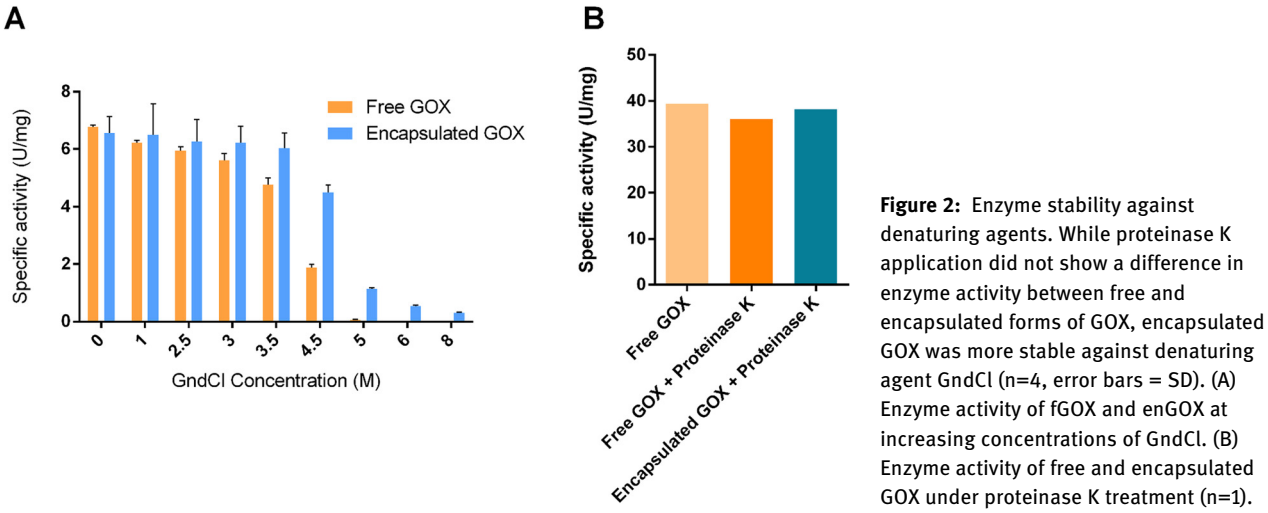


Figure 2: Enzyme stability against denaturing agents. While proteinase K application did not show a difference in enzyme activity between free and encapsulated forms of GOX, encapsulated GOX was more stable against denaturing agent GndCl (n=4, error bars = SD). (A) Enzyme activity of fGOX and enGOX at increasing concentrations of GndCl. (B) Enzyme activity of free and encapsulated GOX under proteinase K treatment (n=1).

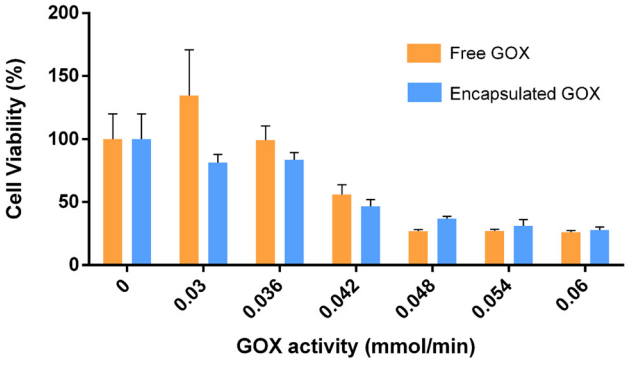


Figure 3: The effect of the enzyme on cancer cell viability. Both free and encapsulated glucose oxidase inhibited the viability of MCF-7 breast cancer cells in an activity dependent manner. Viability of cells not receiving a treatment considered as 100% (n=4, error bars = SD).

GOX inhibits viability of MCF-7 cancer cells. – Both fGOX and enGOX inhibited viability of MCF-7 breast cancer cells in an activity dependent manner (Figure 3). Encapsulation process did not seem to reduce the ability of GOX to inhibit cancer cell viability as both forms affected the cells similarly. All graphs were drawn using GraphPad Prism 6.0.

Discussion

GOX acts as an ideal anti-cancer therapeutic enzyme as its substrate, glucose, is essential for survival of cancer cells and its product, hydrogen peroxide, is toxic to them [10]. As a result, GOX can cause a multiple-hit attack on cancer cells blocking macromolecule synthesis and ATP production as well as disrupting the redox homeostasis. Even though glucose-depletion based starvation-like therapies has been previously demonstrated to be effective [11, 12], the use of GOX in cancer treatment much like all the other therapeutic enzymes is limited as protein activity is quickly lost after administration due to denaturing and degrading defense mechanisms of the human body such as digestive enzymes and neutralizing antibodies [13]. Researchers rely on different encapsulation and immobilization methods to overcome this shortcoming including the use of biotinylated self-assembled vesicles [14] and nanocarriers [15]. Another method of immobilization, polyethylene glycol conjugation to the enzyme (PEGylation), has previously been demonstrated to prolong the circulating half-life of asparaginase therapeutic enzyme without decreasing its activity and it prevents potential allergic reactions [16].

In current study we embraced a similar approach and encapsulated the enzyme with a thin and permeable [17] acrylamide coat which would protect the enzyme from degrading and denaturing agents without limiting the access of the enzyme to the substrate or the movement of the products away from the enzyme. Acrylamide hydrogels are being extensively used in cosmetics and reconstructive medicine due to their biocompatibility [18]. However, this is not an indicator of its biodegradability and stability as a drug carrier and future studies should be designed to address this.

Moreover, with this method each enzyme molecule can be coated separately leading to the formation of single enzyme nanoparticles and the size of these nanoparticles can be adjusted by altering the concentration of the

polymerizing agent acrylamide. Having nanoparticles of desired sizes is crucial for nanoparticle based targeted cancer therapies due to leaky vascular architecture of the microenvironment where nanoparticles of certain size can be trapped and accumulated [19].

Conclusion

GOX itself is very resistant against proteolytic degradation by proteases like trypsin, papain and pepsin [20] and encapsulation did not provide additional protection in our experiments. However, encapsulation did provide protection against denaturation without reducing its activity, which would prolong half-life of the enzyme when administered as a drug. GOX proved to be highly effective in inhibiting MCF-7 breast cancer cell viability possibly via depleting glucose and inducing oxidative damage, and encapsulation did not hamper this effect. Further *in vitro* and *in vivo* studies are required to investigate its action on non-neoplastic cells, to prove the molecular mechanism of action of the enzyme (just glucose consumption, just hydrogen peroxide production or both) and possible internalization of the particle as well as to demonstrate its usefulness in animal models. Since *in vivo* is a more challenging environment for enzyme-based therapeutics due to digestion process and antibodies, we believe the superiority of enGOX over the fGOX may be more apparent in such setting.

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