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Effect of formulation factors on the bioactivity of glucose oxidase encapsulated chitosan–alginate microspheres: *In vitro* investigation and mathematical model prediction

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HIGHLIGHTS

- We prepare glucose oxidase encapsulated polymer microspheres of various sizes.
- We develop a mathematical model to predict enzymatic reaction and release kinetics.
- We investigate the effect of formulation factors on bioactivity of microspheres.
- Increasing release rate and bioactivity of H_2O_2 in cancer cells with enzyme level.
- Computer simulation predicts effect of formulation factors on H_2O_2 release rate.

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ABSTRACT

Higher reactive oxygen species (ROS) levels in cancer cells than normal cells have long been recognized, which makes cancer cells more susceptible to excess ROS. Thus oxidation (also called pro-oxidant) therapy has been explored as new cancer therapy regimens. To produce additional ROS, *e.g.* H_2O_2 *in situ* within tumor, we encapsulated glucose oxidase in chitosan-coated alginate–calcium microspheres (GOX-MS) for locoregional treatment and demonstrated its efficacy against cancer cells *in vitro* and *in vivo*. Owing to the complex biological functions of ROS, the production rate and amount of H_2O_2 are critical to achieve therapeutic benefits without causing normal tissue toxicity. This work was therefore intended to investigate the effect of formulation factors, *e.g.*, particle size and enzyme loading level, on the H_2O_2 generation kinetics and bioactivity of GOX-MS. *In vitro* studies revealed that, at the same GOX loading levels, smaller GOX-MS (20 μm in diameter) generated more H_2O_2 and killed more cancer cells than the larger (140 μM) GOX-MS. A mathematical model including simultaneous diffusion and enzymatic reaction was developed to describe the release kinetics of generated H_2O_2 from a GOX-loaded spherical polymeric matrix. Profiles of species concentration, pH, polymer volume fraction and solute diffusivity inside the microspheres were numerically calculated. The model predicted H_2O_2 release profile was verified by the experimental data. Numerical analysis predicted quantitatively how H_2O_2 generation increased with increasing GOX loading inside the GOX-MS, glucose concentration in the external solution, or with decreasing particle size. The findings and methodology presented herein are useful for optimizing the design of GOX-MS and applicable to understanding and development of other enzyme-encapsulated polymer microspheres.

1. Introduction

It has long been recognized that cancer cells generate more reactive oxygen species (ROS) than normal cells due to their high proliferation rate and altered metabolic pathways (Davies, 1995; Sundaresan et al., 1995; Beckman and Ames, 1997; Halliwell and Gutteridge, 1984; Bladier et al., 1997; Fruehauf and Meyskens, 2007). Because of this characteristic, cancer cells are believed to be more sensitive to excess amount of ROS. However, ROS functions like a double-edged sword (Lopez-Lazaro, 2007). At low levels, ROS participates in signal-transduction and promotes cell growth. At high levels, ROS causes detrimental damages to cells such as lipid peroxidation and DNA damage causing cell death. Antitumor activity of H_2O_2, one member of ROS family, was discovered as

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early as in 1950s (Makino and Tanaka, 1953; Green and Westrop, 1958; Sugiura, 1958; Mealey, 1965; Nathan and Cohn, 1981). Further understanding of double functions of ROS has led to the exploration of a new cancer therapeutic regimen, so called oxidation therapy or (pro-) oxidant therapy, which has gained increasing interest (Pelicano et al., 2004; Verrax et al., 2008; Trachootham et al., 2009; Cabello et al., 2007; Nogueira and Hay, 2013; Gorrini et al., 2013; Glasauer and Chandel, 2014).

Compared to other ROS members, such as superoxide anion (O_2^-), and hydroxyl radical ($\cdot OH$), H_2O_2 is more desirable as a therapeutic agent for its better capability of penetrating cell membranes. Nonetheless, H_2O_2 still has a short life time *in vivo* due to decomposition by antioxidant enzyme catalase and quick clearance. Therefore, H_2O_2-producing enzymes, such as bovine serum amine oxidase and D-amino acid oxidase, have been studied by various groups (Yagi, 1971; Konno and Yasumura, 1992; Ben-Yoseph and Ross, 1994; Sawa et al., 2000). However, the low *in vivo* stability of native enzymes limited their application. Therefore, modification of the enzymes using polymer conjugations or microencapsulation has been pursued with improved stability and prolonged activity (Averill-Bates et al., 2005; Demers et al., 2001; Liu et al., 2007, 2009, 2010).

Chitosan coated alginate–calcium microspheres with a matrix structure have been developed in our group to encapsulate glucose oxidase (GOX) and demonstrated to be effective in killing P-glycoprotein overexpressing cancer cells *in vitro* and inhibiting tumor growth *in vivo* (Liu et al., 2007, 2009, 2010). As illustrated in Fig. 1, when the GOX-loaded microspheres (GOX-MS) are placed in a cell culture medium or physiological fluid containing glucose, glucose and oxygen diffuse into the GOX-MS and react in the presence of GOX. The products, gluconic acid and H_2O_2 then diffuse out to the surrounding medium. Only the released H_2O_2 could reach cancer cells and exert anticancer activity. Our previous results indicated that the cytotoxicity of encapsulated GOX is correlated to the amount of H_2O_2 produced and detected in the medium.

For quick diffusion-in of the reactants and diffusion-out of the products, in particular the anticancer species H_2O_2, the microsphere matrix should be largely permeable to both reactants and products. Meanwhile, they should not allow the enzyme GOX to leak out. The previously designed chitosan-coated alginate microspheres in our laboratory are well suited for this purpose. The parent calcium-conjugated alginate microspheres were prepared using an emulsification–internal gelation method (Liu et al., 2007). After GOX loading into the microspheres, chitosan in a dilute solution was introduced to form 3-dimentional networks with alginate by ionic complexation. The resultant hydrogel microspheres are highly swollen and soft with Young's modulus close to blood cells (Kim et al., 2009, 2010). However, the chitosan-reinforced microspheres allow minimal GOX diffuse out, while permitting small molecules diffusion in and out readily. Owing to the presence of ionizable carboxylic groups in alginate chains, the GOX-MS showed pH-dependent swelling and softness with higher swelling in pH 7.4 buffer than in water with a pH close to 6 (Kim et al., 2010). Because the amount and rate of H_2O_2 generated by GOX-MS are important for achieving treatment efficacy while minimizing systemic toxicity, we investigate herein how formulation factors, such as particle size, GOX loading, and glucose concentration, would influence H_2O_2 production by experiments and model prediction, for the rational design of GOX-MS formulation. Following our previous work in which mathematical models were developed to predict the behavior of glucose sensitive cationic or nanocomposite membranes (Abdekhodaie and Wu, 2005, 2009), we developed a model for bi-directional diffusion of reactants and products as well as enzymatic reaction of glucose in a spherical matrix. The concentration profiles of various species inside the GOX-MS were calculated and the kinetics of H_2O_2 release into the surrounding medium was predicted. Owing to the gradual release of gluconic acid, the microdomain pH in the matrix can be acidic (Abdekhodaie and Wu, 2005, 2009; Huang et al., 2008). Therefore this model also took into consideration pH-dependent polymer swelling and solute diffusivity in the hydrogel matrix. Having verified by the experimental data, the model was applied to analyze quantitatively the dependence of H_2O_2 release kinetics on various formulation factors. The bioactivity and cytotoxicity of GOX-MS in relation to particle size and GOX loading level were also studied *in vitro*.

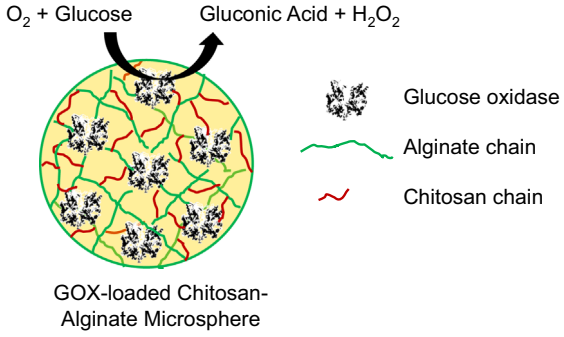

Fig. 1. Schematic diagram illustrating the structure of a glucose oxidase loaded chitosan–aliginate microsphere and hydrogen oxide production. Glucose and oxygen in the medium diffuse into the microsphere and react, as catalyzed by glucose oxidase, to produce gluconic acid and hydrogen peroxide. The latter then diffuse out of the microsphere into the medium.

2. Experiments

2.1. Materials

Alginate (sodium salt, 1.5% solution, medium viscosity), glucose oxidase (Type X-S, 190 units/mg, from Aspergillus niger), glucose, Span 80 and Tween 80 were purchased from Sigma (St. Louis, MO, USA). Chitosan (MW=62 kDA) purchased from Fluka (Buchs, SG, Switzerland) was modified by potassium persulfate (KPS) degradation to prepare 1% chitosan. Calcium carbonate, light mineral oil and acetic acid were purchased from Fisher (Fairlawn, NJ, USA). Phosphate-buffered saline (PBS) pH 7.4 was purchased from Invitrogen (Burlington, ON, Canada).

2.2. Preparation of chitosan-complexed GOX-loaded alginate microspheres

The emulsion-internal gelation method was employed for the synthesis of calcium–alginate microspheres (Liu et al., 2007). To 6 mL of 1.5% alginate solution, 20 mg of calcium carbonate powder was added. The mixture was sonicated in an ultrasonic bath for 4 min and then introduced to a solution composed of 30 mL of light mineral oil, 450 μL of Span 80 and 60 μL of acetic acid stirred at 770 rpm at room temperature. After 5 min, the stirring speed was decreased to 400 rpm and then 120 mL of DDI water was added to the emulsion. Following stirring for another 10 min, the emulsion was stirred slowly with a magnetic stirring bar for 60 min. The supernatant and foam residue were removed and the precipitate was rinsed with 200 mL of DDI water and 2 mL of Tween 80. The final microspheres were collected after repeatedly rinse with DDI water. Then 500 μL of microspheres were transferred to 500 μL of 500 μg/mL glucose oxidase in pH 4 acetate buffer. The loading experiment was carried out for 30 min at 4 °C, followed by incubation of the GOX loaded microcapsules with 1% chitosan solution (w/v) in 0.3% (v/v) acetic acid for 10 min. At the

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