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# Charge-selective fractions of naturally occurring nanoparticles as bioactive nanocarriers for cancer therapy

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ABSTRACT

A carnivorous fungus, *Arthrobotrys oligospora*, has been shown to secrete nanoparticles. In the present work, the potential of two charge-selective fractions of fungal nanoparticles (FNPs) as bioactive nanocarriers in cancer therapy is explored by investigating their immunostimulatory activities, cytotoxic mechanisms and in vitro immunochemotherapeutic effects. A surface charge-selective fractionation procedure to purify crude FNPs has been established, and two FNP fractions (i.e. FNP1 and FNP2), with different surface charges and similarly reduced diameters of 100–200 nm, are obtained. Both FNP fractions enhance the secretion of multiple proinflammatory cytokines and chemokines from macrophages and splenocytes. However, FNP2 has stronger cytotoxicity than FNP1. It is FNP2 not FNP1 that could clearly inhibit cell proliferation by inducing apoptosis and arresting cells at the sub G0/G1 phase. Both the FNP fractions can form pH-responsive nanocomplexes with doxorubicin (DOX) via electrostatic interactions. For direct cytotoxicity, DOX–FNP2 complexes demonstrate higher activity than DOX against multiple tumor cells, while DOX–FNP1 complexes show weaker activity than DOX. Interestingly, in a co-culture experiment where splenocytes are co-cultured with tumor cells, both DOX–FNP complexes demonstrate higher cytotoxicity than DOX. In conclusion, this work proposes a combined therapeutics for cancer treatment using charge-selective fractions of FNPs as bioactive nanocarriers.



## 1. Introduction

Cancer is a leading cause of death worldwide, and it is estimated that 13.1 million will die of this disease in 2030 [1]. Chemotherapy is generally regarded as the first-line approach for the treatment of malignant cancer [2,3]. To avoid the emergence of systemic toxicity and therapy resistance, it is essential to develop new treatment modalities with multiple mechanisms of cell killing in tumors, i.e. combined therapy. A few combination therapies using engineered nanoparticle-based delivery systems, including nanoparticles [4,5], liposomes [6–8] and macromolecular conjugates [9] in conjunction with different chemical drugs and immune-stimulants, have been reported [10]. However, among these nanoparticle-enhanced combinatorial therapies, few engineered biomaterials play the role of immunostimulants or adjuvants. They are usually inert biomaterials, simply conjugated or encapsulated with an immunostimulatory agent and a chemodrug in the combined antitumor therapy.

Naturally occurring nanoparticles are an alternative source for producing bioactive biopolymer-based nanoparticles with diverse chemophysical properties and biofunctions. The use of naturally occurring organic nanoparticles and biomimetic/bioinspired nanomaterials in medicine has drawn increasing interest in recent years. It is anticipated that study of naturally occurring nanoparticles will provide significant insight into the development of bioactive nanomaterials for cancer treatment. In 2012, our group first discovered that nanoparticles secreted from a carnivorous fungus, *Arthrobotrys oligospora*, had promising properties as immunostimulatory and antitumor agents for cancer treatment [11]. *A. oligospora* is a representative flesh eater in the fungal kingdom. It can develop into specialized 3-D adhesive traps for capturing, penetrating and digesting free-living nematodes in diverse environments [12]. A scalable and robust platform was developed to produce these fungal nanoparticles (FNPs) from a sitting drop culture system [11]. From this platform, the FNPs collected by a washing-dialysis procedure showed a size of 200–300 nm in diameter measured by scanning electron microscopy (SEM)/atomic force microscopy (AFM), and 300–400 nm in aqueous suspension

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measured by dynamic light scattering (DLS) [11]. From the perspective of mere passive tumor targeting in vivo, the upper bound size of the nanoparticles participating in the enhanced permeability and retention (EPR) effect is believed to be ~400 nm [13], and an effective drug carrier for in vivo cancer treatment should have a diameter of <200 nm considering the multiple factors in vivo, such as limited fenestration size of the leaky vasculature in tumors, and rapid systemic clearance [14].

Thus, in order to effectively utilize these FNPs as drug carriers for chemical drug delivery into the tumor tissue in vivo, we need to further purify these naturally occurring FNPs to reduce their average particle size without compromising their bioactivities, such as immunostimulation and cytotoxicity. For such a purpose, we have established a surface charge-selective fractionation approach to purify the crude FNPs secreted from the sitting drop culture system. At the same time, the physicochemical properties of the newly isolated FNP fractions are characterized, and their potential use as bioactive nanocarriers in cancer therapy is finally explored by investigating their bioactivities against distinct immunocytes and tumor cells, as well as the combined immunochemotherapeutic effects in an in vitro co-culture system.

## 2. Materials and methods

### 2.1. Chemicals, fungus and cell lines

*A. oligospora* (ATCC 24927), A549 human non-small-cell lung cancer cells (CCL-185) and RAW 264.7 murine macrophages (TIB-71) were obtained from the American Type Culture Collection (Manassas, VA). B16BL6 murine melanoma cells, MCF-7 human breast tumor cell line, and multidrug resistant cell line MCF-7/ADR were obtained from the National Cancer Institute-Central Repository (Frederick, MD). Splenocytes, derived from C57BL/6 mice, were purchased from the Allcells Company (Emeryville, CA). HEPES, 1,9-dimethyl-methylene blue (DMMB), chondroitin sulfate (CS), Sephadex G75, DEAE-cellulose, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich (St Louis, MO). Doxorubicin hydrochloride (DOX) was purchased from Abcam (Cambridge, MA). LysoTracker Green DND-26 and Hoechst 33342 were purchased from Invitrogen Life Technologies (Grand Island, NY). Fetal bovine serum, DMEM medium and RPMI 1640 medium were purchased from Mediatech (Manassas, VA). Penicillin (10,000 units $ml^{-1}$)–streptomycin (10,000 μg $ml^{-1}$) solution was obtained from MP biomedicals (Solon, OH).

### 2.2. Arthrobotrys oligospora culture and FNPs fractionation

*A. oligospora* was cultured in the sitting drop culture system proposed early [11] with some modifications to scale-up production and improve purification quality. Briefly, conidia suspension (about 1000–2000 conidia in 200 μl) was inoculated into the media droplet and incubated at 25 °C for 7 days. The isolation procedure was shown in Fig. 1A. First, the mycelia developed on the cover slip were washed over 10 times using distilled water. The collected water containing nanoparticles were then filtered through a 0.2 μm syringe filter (cellulose acetate, VWR, Radnor, PA). The FNPs were then desalted by a size exclusion chromatography (SEC, Sephadex G75) column [15]. The desalted FNPs is designated as FNP0, which is a crude sample. To further purify the FNP0, weak anion-exchange (WAX) chromatography on DEAE-cellulose was performed [16]. The DEAE-cellulose columns were then eluted in a stepwise fashion with 0.1, 0.2, 0.3, 0.5 and 1.0 M NaCl. As reported in a previous study [11], glycosaminoglycan (GAG) has been determined to be one of the main components in the FNPs. Thus, the colorimetric assay ($\lambda_{525nm}$) for GAG with 1,9-dimethyl-methylene blue was used to monitor the FNP fractions in the eluates from the SEC or DEAE-cellulose column. The elution profiles of the FNPs, reflected from GAG concentration, were plotted vs. elution volumes. The collected peaks containing FNPs from WAX column were subjected to the Sephadex G-75 column for desalting. The desalted FNP fractions were concentrated to final volume of 150 μl using a centrifugal filter tube (Amicon Ultra-15 100K, Merck Millipore, Ireland).

### 2.3. Characterization of FNP fractions

To characterize nanomorphology and particle size of the FNP fractions, the samples were analyzed using AFM (MFP-3D, Asylum Research, Santa Barbara, CA) with an IGOR Pro control system. Briefly, 10 μl of the particle suspension was air-dried on a glass cover slip, and imaged in AC mode at room temperature using a silicon probe PPP-NCHR-20 (Nanosensors™, Neuchatel, Switzerland) with a cantilever spring constant of 42 N $m^{-1}$ and a resonance frequency of 330 kHz. The nanoparticle samples were also analyzed by DLS and electrophoretic light scattering (ELS) to determine the size distribution and zeta potential in aqueous suspension using a Zetasizer Nano (Malvern Instruments Ltd., Worcestershine, UK) with a He–Ne laser (wavelength of 633 nm) and a detector angle of 173°. All samples were measured in triplicate. To qualitatively determine the chemical components in the nanoparticles, SDS–PAGE was used, and then the GAG, neutral polysaccharides and proteins in the nanoparticles were stained using Alcian blue, PAS reagents and silver staining reagents, respectively. To quantitatively determine the chemical components in the nanoparticles, total amounts of polysaccharides were measured using anthrone-sulfuric acid assay [17]. The amount of GAG in each sample was determined by a proteoglycan detection kit (1,9-dimethylmethylene blue, Astarte Biologics, Redmond, WA) [11], and the uronic acid in the nanoparticles was determine using carbozole assay [18]. Meanwhile, the concentration of proteins in the samples was quantitatively determined by the BCA protein assay (Pierce, Rockford, IL) following the manufacturer's instructions.

### 2.4. Immunostimulatory activity

The mouse macrophage RAW 264.7 cells (ATCC TIB-71) and splenocytes derived from C57BL/6 mice were cultured in DMEM and RPMI 1640 culture media, respectively. Both media were supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in 5% $CO_2$. The cells were plated in 12 well plates at a density of $5 \times 10^6$ cells $ml^{-1}$, treated with the FNPs at the GAG concentration of 5 μg $ml^{-1}$. After a 24 h incubation, the supernatants were collected for ELISArray. Mouse common cytokines and chemokines multi-analyte ELISArray kits (SABiosciences Corporation, Frederick, MD) were used to determine 12 cytokines (IL-1A, IL-1B, IL-2, IL-4, IL-6, IL-10, IL-12, IL-17A, IFNγ, TNFα, G-CSF and GM-CSF) and 12 chemokines (RANTES, MCP-1, MIP-1a, MIP-1b, SDF-1, IP-10, MIG, Eotaxin, TARC, MDC, KC and 6Ckine) in the supernatants following the manufacturer's instructions. The concentration of nitric oxide (NO) in the supernatants of both cells treated with the FNP samples were also determined using Griess assay, as described elsewhere [19].

### 2.5. MTT assay

The cytotoxicity of the purified FNP fractions and the DOX–FNP complexes against four cancer cell lines (A549, B16BL6, MCF-7 and MCF-7/ADR cells) was evaluated by MTT assay [15,20]. Biocompatibility of the purified FNP fractions toward mouse fibroblast NIH3T3 cell was also measured through MTT assay. Briefly, 8000–10,000 cells were plated in 96-well plates in 100 μl culture