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Enhancement of Immunomodulatory and Anticancer Activity of Fucoidan by Nano Encapsulation

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Abstract The aim of the present study was to prepare nanosample of fucoidan using lecithin as encapsulated material and to investigate the anticancer and immunomodulatory activity of nanoparticle *in vitro*. The nanoparticles have been characterized by dynamic light scattering (DLS) and transmission electron microscopy (TEM). Confocal microscopy confirmed the internalization of the fucoidan conjugates into the immune cells. The uptake of nanoparticles was confirmed with confocal microscopy demonstrating their localization in the cells. The anticancer activity was increased over 5-10% in different cancer cells of fucoidan nanoparticle as compare with fucoidan. The human B and T cells growth and the secretion of interleukin-6 and tumor necrosis factor-α from B cell were also improved by fucoidan nanoparticle because of the rapid absorption of nanoparticle into the cells as compare to fucoidan. At 0.6 mg/mL concentrations, the fucoidan nanoparticle showed better activity than 1.0 mg/mL concentration in T cell growth because the cells reached their saturation capacity. When the fucoidan was encapsulated in lecithin, its anticancer as well as its immunomodulatory activity proved to be superior from that of itself in pure form.

Keywords: fucoidan, nanoparticle, lecithin, confocal, bioactivity

Introduction

Brown seaweeds (Phaeophycophyta) are known to produce different polysaccharides, namely alginates, laminarans, and fucoidans (1). The latter polysaccharides usually contain large proportions of L-fucose and sulfate, together with minor amounts of other sugars like xylose, galactose, mannose, and glucuronic acid (2). Sulfated polysaccharide in seaweed contains diverse biological activity in potential medicinal value, such as anticoagulant, antithrombotic, anti-inflammatory, antitumor, contraceptive, antiviral, and antioxidant (3-8). Several studies also report that fucoidan is one of legends for macrophage scavenger receptors (MSR) and induced cytokine expression such as tumor necrosis factor (TNF)-α, interleukin (IL)-1α, IL-8, IL-10, and IL-12 through MSR (9-12). They have particularly been described as inhibitors of the replication of several enveloped viruses, as human immunodeficiency virus, herpes simplex virus, and human cytomegalovirus (13,14).

Encapsulation is a process in which thin films, generally of polymeric materials is applied to little solid particles, liquid, or gases droplets. This method is used to trap active components and release them under controlled conditions. Several materials like carbohydrate, amino acids, vitamins, minerals, antioxidants, colorants, enzymes, and sweeteners have been encapsulated in the food industry and pharmacology. Liposomes as a drug delivery system can improve the therapeutic activity and safety of drugs, mainly by delivering them to their site of action and by maintaining therapeutic drug levels for prolonged periods of time (15-17). Encapsulating a sufficient amount of the therapeutic agent is one of the most desirable properties for their usage (18-20). Development of stable liposomes is a prerequisite for its exploitation in the delivery of therapeutic molecules (21,22). Liposomes prepared from commercially available phospholipids showed lower stability than synthetic or purified phospholipids (23,24).

In the present study, the fucoidan nanoparticle was prepared and characterized by using lecithin (phosphatidylcholine) as encapsulating material and investigated the effect of encapsulated fucoidan nanoparticle in anticancer and immunomodulatory effect *in vitro*.

Materials and Methods

Materials Fucoidan extracted from *Fucus vesiculosus* was obtained from Sigma-Aldrich (St. Louis, MO, USA) and used without any purification. The following chemicals were also obtained from Sigma-Aldrich: lecithin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), ethylenediamine tetraacetic acid sodium salt (Na-EDTA), trichloroacetic acid (TCA), and Hepes buffer. All other chemicals were of the highest analytical grade and were purchased from common sources.

Cell culture The human lung carcinoma (A549), human hepatocellular carcinoma (Hep3B), human breast adenocarcinoma (MCF-7), human gastric cancer cell line (AGS), human embryonic kidney cell (HEK293), human T

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cell (Jurkat), human B cell (Raji), and human NK cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The A549, AGS, macrophage, human B and T cells were maintained in RPMI-1640 supplemented with 10% fatal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere of 50 μg/mL CO_2 at 37°C. The HEK293, MCF-7, and Hep3B cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, Mediatech Cellgro, Herndon, VA, USA).

Preparation and characterization of nanoparticle Lecithin-fucoidan complex nanoparticles were prepared as described previously (25). Lecithin 100 mg was dissolved in 25 mL choloform and prepared the lipid layer using rotary evaporator at 37°C. Fifty mL of aqueous solution of fucoidan (1%, w/v) was added to the dried lipid and hand shaken (swelling) and suspension was sonicated for 20 min. Lecithin nanoparticles were characterized by dynamic light scattering (DLS). The particle size and stability measurements were carried out on a 90 plus particle size analyzer, from Brookhaven Instruments Corp. (BIC, Austin, TX, USA). The light source was a 35 mW He-Ne laser emitting monochromatic wavelength of 632.8 nm, which was focused on the sample and scattered light was detected by a photo-multiplier tube (Hamamatsu, Hamamatsu, Japan). The samples for transmission electron microscopy (TEM) were obtained by dispersing a small drop of the suspension with a much lower concentration onto a copper grid pre-coated with amorphous carbon, and was dried in drying oven at 40°C for 1 day before observation. TEM pictures were taken working at 200 kV (26).

Determination of *in vitro* cytotoxicity against human cancer cell lines *In vitro* cytotoxicity assays were performed against A549, MCF-7, Hep3B, AGS, and HEK 293 cell lines. All cell lines were incubated in tissue culture flasks in desired media in a humidified atmosphere at 37°C with 5% CO_2, trypsinized (trypsin-EDTA, Gibco, Grand Island, NY, USA) cell cultures were washed with media and diluted to deliver 1×10^4 live cells/mL aliquot in each of 96-well plate. The plates were kept in incubator for 24 hr. To determine cell survival after exposure to different concentrations of the individual plant extracts for 48 hr, the sulforhodamine B (SRB) cytotoxicity assay was performed according to Mitry *et al.* (27) with some modification. In each well of the 96-well plate, 100 μL of 20% ice-cold trichloroacetic acid solution was gently layered on top of the medium overlaying with the cells. The plates were then incubated for 60 min at 4°C. Wells were rinsed 5 times with tap water and then cells were stained with 0.4% SRB solution (50 μL stain/well) for 15 min at room temperature. SRB staining solution was poured off; wells were rinsed 5 times with 1% acetic acid to remove unbound dye, and left to air dry. The bound SRB dye was then solubilized by adding 100 μL/well of Tris-base solution, and plates were placed on a plate shaker for 1 hr at room temperature. Plates were then read at 540 nm wavelengths, using a microplate reader (THERMO max; Molecular Devices, Sunnyvale, CA, USA) and the results were expressed as a percentage of control values.

***In vitro* proliferation human B and T cells** The growths of human B and T cells according to concentration of fucoidan and nanosample were determined by MTT assay after treatment with different concentration of fucoidan and nanosample. Based on the method of Oka *et al.* (28) the MTT assay measures the metabolism of 3-(4,5-dimethylthiazol-2yl)-2,5-biphenyl tetrazolium bromide to form an insoluble formazan precipitate by mitochondrial dehydrogenases only present in viable cells. Twenty μL of MTT solution was added to the 100 μL medium in each well of the 96-well plate, and the plate was incubated at 37°C, for 4 hr. Finally, 100 μL isopropanol/HCl (0.04 M HCl) was added per well, the plate was shaken for 30 min on a shaker and the absorbance at 630 nm wavelength was measured using the microplate reader.

Secretion of cytokine from B and T cells Secretion of cytokines were quantified by measuring the amounts of IL-6 and TNF-α using IL-6 and TNF-α kits from Chemicon (Temecula, CA, USA). After adjusting the cell concentration to 1-2 $\times10^4$ cells/mL, 900 μL the cell concentrates were then placed into 24-well plates and cultured for 24 hr (37°C, 5% CO_2). Then, 100 μL of 0.5 mg/mL cell were again cultured (37°C, 5% CO_2). The sample was centrifuged to obtain the supernatant, which was used to read the absorbance at 450 nm using a microplate reader. The amounts of cytokines were measured using the optical density (OD) values of the standards (29).

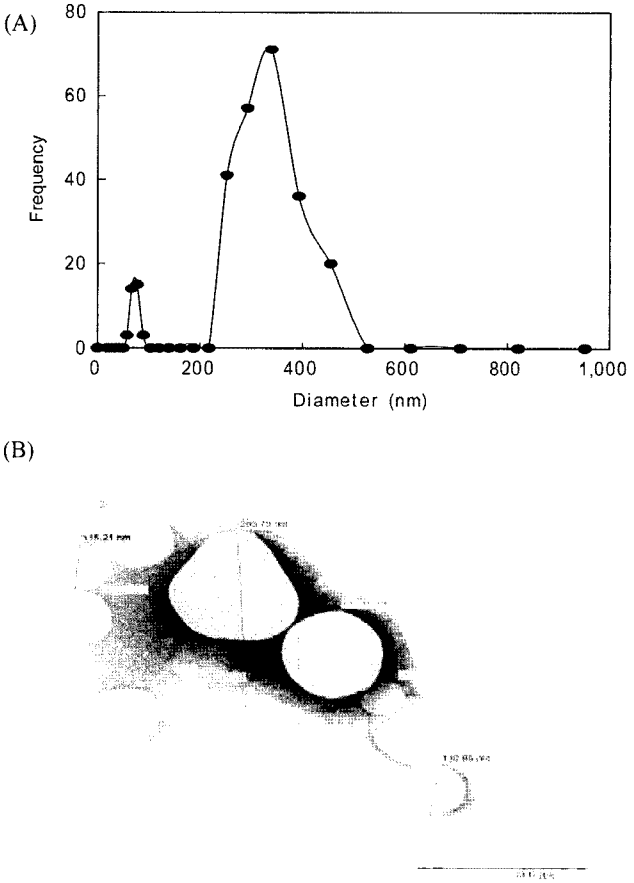

Fig. 1. Characterization of nanoparticles. (A) Size distribution of lecithin liposome containing fucoidan, measured by the DLS technique, (B) TEM micrographs of lecithin liposome (50,000×).

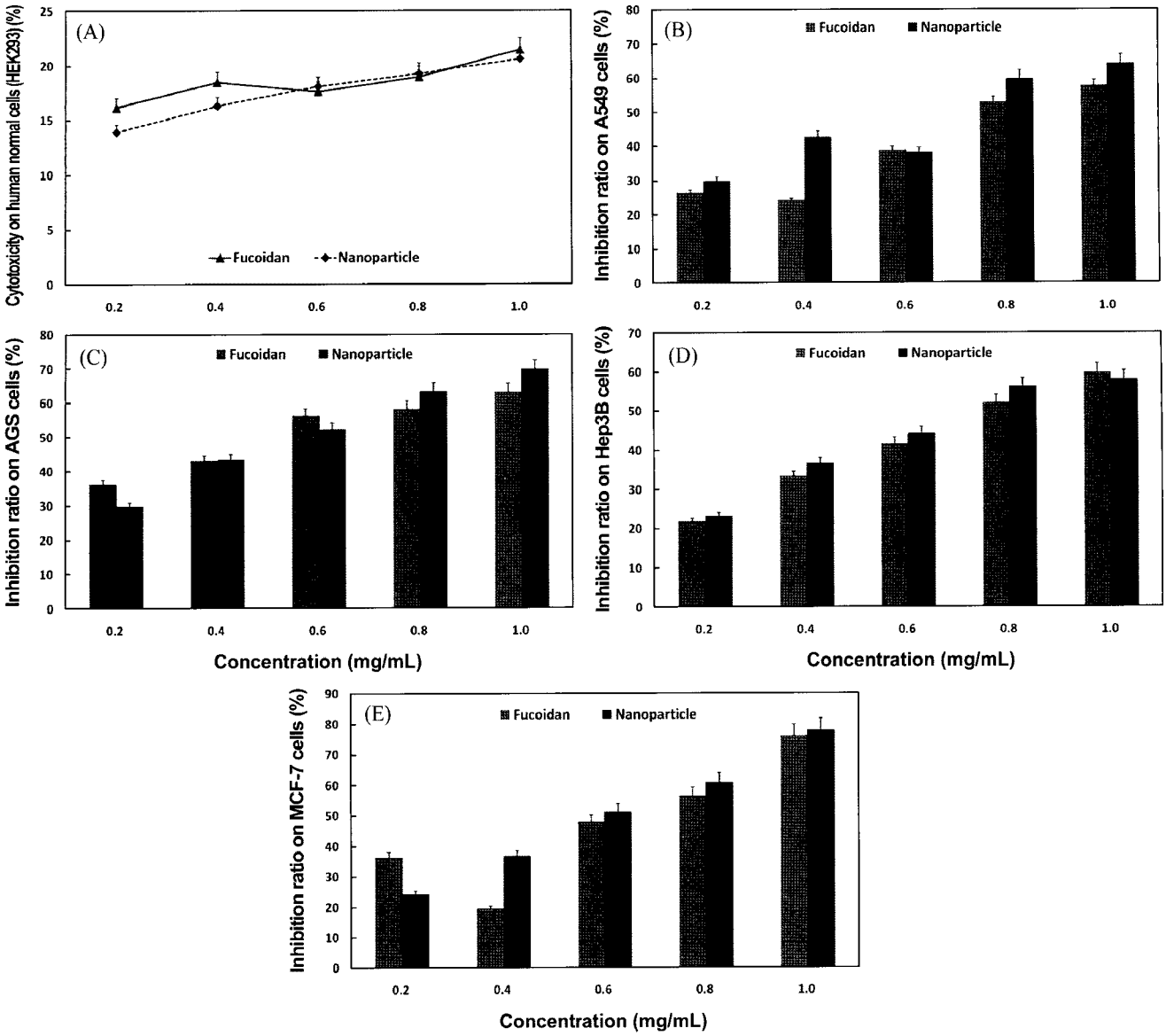

Fig. 2. Inhibition ratio of growth of (A) HEK293, (B) A549, (C) AGS, (D) Hep3B, and (E) MCF-7 in adding different concentration fucoidan and nanoparticle of fucoidan. The line or bar represents the mean±SD of 3 experiments, performed in duplicate.

Enhancement of human NK cell growth The NK-92MI cell line (ATCC, CRL-2408) was diluted to 2×10^7 cells/mL using 2 mM L-glutamine, 0.2 mM myoinositol, 20 mM folic acid, 0.1 mM 2-mercaptoethanol, 12.5% FBS, and 12.5% horse serum (MyeloCult) in MEM medium. While culturing the human B cells in T-25 Flask, the degree of proliferation was observed after placing each sample at a concentration of 0.5 mg/mL. It was sub-cultured 3 to 4 times and centrifuged to obtain the supernatant. After 900 μL of the NK-92MI cell line was aliquoted into 24-well plates at $4\text{-}5\times10^4$ cells/mL, 100 μL of the supernatant from B cells was placed into the well and cultured for 48 hr. Finally, the growth of NK-92MI cell was observed for 6 days using a cell counter (30).

Preparation of liposome for confocal microscopy Formation of lecithin-calcein complex with fucoidan nanoparticles were prepared as described previously. In brief, 20 mg of lecithin was dried by using evaporator and then, dried lipid film was hydrated by 10 mL of water containing ingredient and calcein (fluorescence dye) and suspension was sonicated for 20 min. Final concentration of calcein is 0.15 mM (Mw of calcein=994.9). Fucoidan nanoparticle accumulation in treated cells was located by confocal microscopy. Briefly, cells (human B cell) from exponential cultures were grown in 12-well plates at a density of 5×10^4 cells/mL. One day later, the cultures were washed and treated with serum-free, RPMI medium containing either calcein-loaded lecithin nanoparticles or free calcein (1 hr incubation). All incubations were carried out at 37°C with an equivalent final concentration of calcein (5 μg/mL). Three dimensional (3D) image reconstructions of calcein-labeled nanoparticles were obtained with a Zeiss LSM 410 confocal laser scanning microscope (Zeiss, Oberkochen, Germany) equipped with a computer-controlled, motorized scan stage. An argon laser for fluorescein isothiocyanate (FITC) excitation at 490 nm was used for imaging. A 510-525 nm band-pass filter for the FITC

emission signal was placed in the front of detectors.

Statistical analysis SAS PC package (SAS Institute, Cary, NC, USA) was used for statistical analysis. Results are expressed as the mean±standard deviation (SD) of triplicates.

Results and Discussion

Particle size distribution analysis The particle size distribution analysis of fucoidan lecithin liposome indicates that the average liposome size is 295 nm and its range from 50 to 500 nm (Fig. 1A). This variation in size may be due to the amplification and elapse time of sonication in the lecithin liposome. The shape of liposomes was almost spherical and the size was less than 500 nm (Fig. 1B). The liposomes were observed as white domains because the samples were pretreated by a negative staining technique.

***In vitro* cytotoxicity against human cancer cell lines** The cytotoxicity of fucoidan and fucoidan nanoparticle were evaluated in human cancer A549, AGS, MCF-7, Hep3B, and normal human kidney cell line HEK293 with the SRB assay. When cells were treated for 2 days with 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mg/mL of fucoidan and fucoidan nanoparticle, the relative cell survival progressively decreased in a dose-dependent manner, as shown in Fig. 2 (A, B, C, D, and E). All the result of fucoidan and fucoidan nanoparticle demonstrated inhibitory activity with the cancer cell line and fucoidan nanoparticle have higher inhibitory activity against all the cancer cell line with the growth inhibition ratio of 77.9, 69.8, 64.2, and 58.0% after 48 hr treatment at 1.0 mg/mL in MCF-7, AGS, A459, and Hep3B, respectively. The fucoidan nanoparticle and fucoidan have almost same cytotoxicity in human kidney cells. Fucoidan has direct anticancer effects on human cancer cells through caspase and ERK pathways (31), therefore no further enhancement of cytotoxicity in normal kidney cell. Because the fucoidan nanoparticle contained 2:1 (w/w) ratio of lipid and fucoidan concentration during encapsulation process, so the nanoparticle has less fucoidan concentration than the crude fucoidan used for this study but nanoparticle showed better activity. The result showed that nanoparticle has better anticancer activity and less toxicity against normal cell line. Therefore nanoparticle formulation is one of the novel drug delivery systems which possess various advantages, including increasing drug or extract solubility, enhancing dissolution rate, ameliorating the bioavailability, and decreasing the dosage required for the same effects, compared with the crude extract (32).

Growth of human B and T cell MTT assay was used, to study the human B and T cell proliferation stimulatory effect of fucoidan and fucoidan nanoparticle in various dosages. The increase of cell viability shows that the fucoidan nanoparticle is not toxic to the immune cell and it potentially modulates the cellular immune response. The fucoidan nanoparticle showed good proliferation effect on human B and T cell at 48 hr (Fig. 3). The highest proliferation effect was observed at 1.0 mg/mL. The proliferation effect increased significantly for both B and T cell in dose dependent manner but the proliferation in T

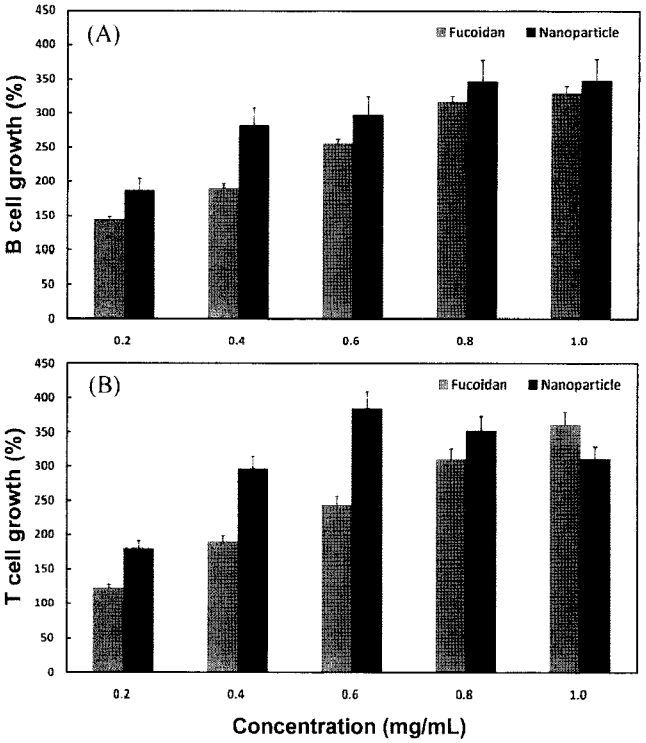

Fig. 3. The growth of B cell (A) and T cell (B) after adding different concentration of fucoidan and nanoparticle of fucoidan. The bar represents the mean±SD of 3 experiments, performed in duplicate.

cell was maximum by fucoidan nanoparticle at 0.6 mg/mL concentration. This was due to the maximum absorption of the nanopaticle at this concentration. Interestingly, the activation of the fucoidan nanoparticle at all the high concentration produced significant proliferation responses.

Secretion of cytokines from B and T cells It is also very important to know the level of secreted cytokines associated with the cell growth. Table 1, illustrates the secretion of both cytokines according to the human B and T cells. The concentration of secreted cytokines from the cell growth must be expressed as specific concentration secreted from each cell, not as a total volume in a culture flask for clearly understanding immunomodulatory effect of the samples. The amount of IL-6, TNF-α released from the cell growth was measured as 7.6×10^{-4} and 7.1×10^{-4} pg/cell by adding 0.5 mg/mL of fucoidan nanoparticle at 6 days. The amount of IL-6, TNF-α released from the T cell growth was measured as 9.1×10^{-4} and 8.9×10^{-4} pg/cell in adding 0.5 mg/mL of fucoidan nanoparticle at 6 days.

Enhancement of NK cells associated with human B cell growth For better understanding the immunomodulatory effect of fucoidan and fucoidan naoparticle, it is also necessary to know the role of B cell growth associated with NK cell growth. NK cell concentration was increased with the addition of amounts of the B cell supernatant containing 0.5 mg/mL of fucoidan or fucoidan nanoparticle. As shown in Fig. 4, human NK cell growth was compared after adding fucoidan and fucoidan nanoparticle treated B cell supernatant and estimated as 12.3×10^4 and 13.6×10^4 cells/mL, respectively, at 6 days. NK cell was gradually improved

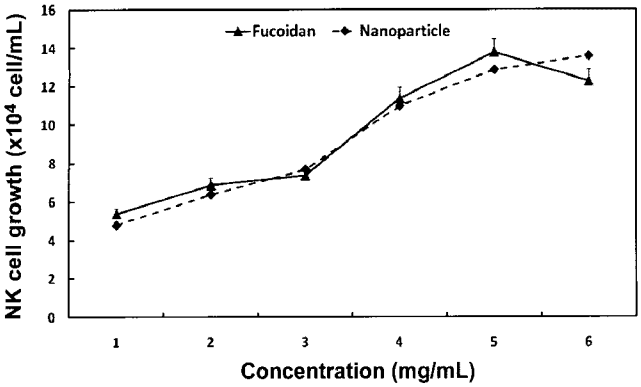

Fig. 4. Effect on NK cell growth by adding the supernatant of B cells treated with 0.5 mg/mL concentration of fucoidan and fucoidan nanoparticle. Each value represents the mean±SD of 3 experiments, performed in duplicate.

as the increase of the addition of the supernatant. This result can support the hypothesis that the cytokines released from human B cells affect the promotion of NK-92MI cell growth.

Confocal microscopy analysis The precise intracellular location of nanoparticles within specific regions of a cell can be confirmed by 3D multispectral confocal microscopy. Calcein loaded nanoparticle localization in human B cells is shown in Fig. 5A and 5B. Within 1 hr incubation, intracellular localization of calcein loaded into lecithin nanoparticles containing fucoidan could be visualized, whereas, free calcein could not enter the cell in 1 hr (Fig. 5C and 5D). A control, without treatment of either free calcein or calcein loaded nanoparticles was also used to see the difference between treated non-treated cells (Fig. 5E and 5F). The nanoparticle uptake was also dependent on the incubation time. The uptake was seen as early as at 1 hr, which increased gradually with the incubation time. Confocal microscopy of the cells exposed to nanoparticles

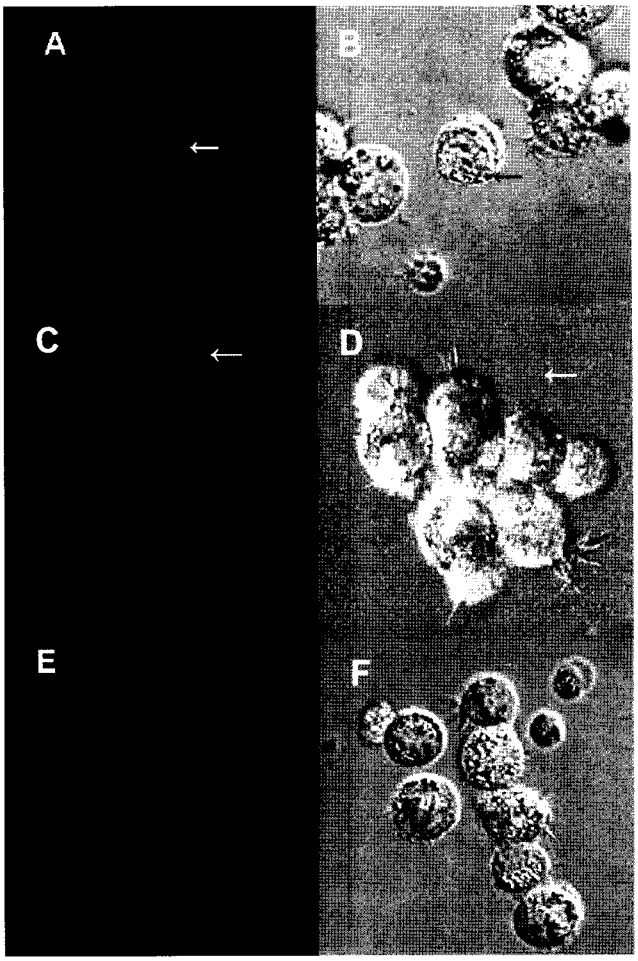

Fig. 5. Confocal microscope photographs of B cells (Raji) after 1 hr growth in media containing fucoidan nanoparticles coated with calcein (A,B), media containing only calcein (C,D), and only media (E,F). Reflectance images were obtained with 647 nm excitation wavelength, and fluorescence images were obtained using 488 nm excitation and 510-525 nm long band-pass emission filter. The arrow showed the localization of dye calcein into the media containing cells.

Table 1. The secretion of IL-6, TNF-α from human B and T cells in adding fucoidan and nanosample (0.5 mg/mL)

Sample	Time (day)	Cytokine emision[1] (10^{-4} pg/mL)			
		B cell		T cell	
		IL-6	TNF-α	IL-6	TNF-α
Fucoidan	1	1.3±0.2*	1.1±0.24*	2.6±0.56**	3.1±0.24**
	2	1.9±0.31*	2.6±0.23*	1.9±0.66*	4.8±0.48*
	3	2.6±0.22**	3.7±0.42*	3.1±0.54*	5.4±0.64*
	4	4.2±0.26***	4.4±0.33***	5.9±0.94*	5.2±0.74*
	5	6.4±0.69 **	5.3±0.42*	8.0±1.34**	6.1±0.66**
	6	7.9±0.74 *	6.5±0.44**	9.1±1.66***	7.3±0.98***
Nanoparticle	1	2.3±0.33**	2.0±0.22*	1.8±0.44*	2.2±0.94*
	2	3.1±0.46 ***	3.9±0.28*	2.7±0.78*	4.6±1.22*
	3	3.9±0.54*	4.8±0.56**	4.0±0.68*	6.9±1.72**
	4	4.7±0.33***	5.9±0.44**	5.8±0.96*	7.3±1.60*
	5	6.9±0.68**	6.7±0.46*	7.9±1.12**	8.4±1.66*
	6	7.6±1.4***	7.1±0.84**	9.1±1.68***	8.9±1.98*

[1]Data values are expressed as mean±SD of triplicate determinations; data showed significant difference from samples are indicated by $*p<0.05$, $**p<0.01$, $***p<0.001$.

demonstrated increased fluorescence activity in the cells with increase in the time of incubation.

Nanotechnology has been introduced into several aspects of food industry including encapsulations and delivery systems which protect and deliver functional food ingredients. This study on fucoidan encapsulated with lecithin revealed successful preparation with efficient encapsulation of fucoidan. Studies using DLS and TEM give evidence of possible interaction between fucoidan and lecithin. This may contribute to enhancing bioactivity by increasing fucoidan permeation. The results clearly demonstrated that fucoidan nanoparticle have better anticancer and immunomodulatory activity at low concentration because of maximum absorption of sample into the cells. The lecithin formulation could therefore be a suitable device for anticancer and immunomodulating agents. The nanotechnology of drug or extract formulation not only enhances the absorption of drugs or extract but it also improves drug therapeutic effectiveness in pharmaceutical research (33). In addition, the lecithin delivery system represents a highly versatile drug carrier, suitable for both *in vitro* and *in vivo* administration. Food-grade materials generally recognized as safe, biocompatible, and biodegradable have the potential to be used for the delivery systems in a wide variety of foods. Our study may provide basic data to establish the procedure for evaluating the safety of the nano-sized foods. Controlling amount and rate of active agents release makes encapsulation suitable for different applications in the food industry.

Acknowledgments

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