Final Scientific Report

Cover Page

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Project Title: Antimicrobial and antioxidant functionalized nanoparticles for enhancing food safety and quality: proof of concept

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Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.
Nanotechnology, phenolic compounds, curcumin, liposome, *Escherichia coli*, *Bacillus cereus*

Abbreviations commonly used in the report, in alphabetical order:

CFN – curcumin-functionalized nanoparticles, MBCD – methyl- β -cyclodextrin, MIC – minimum inhibitory concentration, NHS – N-hydroxysuccinimide, PDA – polydiacetylene

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Final Scientific Report

Publication Summary (numbers)

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Refereed (published, in press, accepted) BARD support acknowledged				
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Invited review papers				
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Abstracts	2			2
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description Cooperation: First project meeting was held in Carbondale in February 2012 with participation of the Israeli Co-PI. It was devoted to discussing the results of the first stage of the project and taking decisions about the most promising phenolic compounds and efficient nanoparticle functionalization methods. The method of nanoparticle assembly and investigation was demonstrated, and a sample of phenolic-functionalized nanoparticles transferred to the Israeli partner. Second project meeting was held in Israel in June 2012 with participation of the US PI. It summarized the accomplishments of both sides by that time and plans for further investigations. Samples of curcumin-functionalized nanoparticles were transferred to the Israeli partners for the final series of experiments and tested in common trial run together with the US partner. The research results were/will be presented at two scientific conventions by joint author teams; the common scientific paper is in preparation.

Patent Summary (numbers)

	Israeli inventor only	US inventor only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
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Abstract

General concept. The reported 1-year study tested the feasibility of preparing antimicrobial and antioxidant nanoparticles functionalized with natural phenolic compounds, as a first step to reach the ultimate goal - improving safety and quality of foods by developing novel antimicrobial and antioxidant food-contacting materials. The secondary objectives of the study were (a) selecting the most promising phenolic compounds, (b) building nanoparticles with the selected phenolics grafted on their surface, and (c) testing antimicrobial and antioxidant properties of these particles. The study was expected to provide a “go/no go” decision as concerning the prospects of phenolic-bound nanoparticles as antimicrobial and antioxidant agents.

Results. In course of the feasibility study, curcumin was chosen as the most promising phenolic compound due to its high antibacterial activity exceeding other tested compounds by at least one order of magnitude. Liposome-type phospholipid/polydiacetylene (PDA) nanoparticles functionalized with curcumin were successfully built. The pitfall of limited curcumin amount that could be covalently bound to the particle surface was circumvented by inclusion of curcumin in the liposome body. It was suggested on the basis of fluorescence spectroscopy that curcumin was bound by hydrophobic forces in the bilayer periphery of the liposomes and therefore might exert a contact effect on microorganisms. The curcumin-functionalized nanoparticles (CFN) were shown to have a strong bactericidal activity towards both Gram-negative (*E. coli*) and Gram-positive (*B. cereus*) bacteria, but only limited effect against yeast. Furthermore, beyond the originally planned objectives, preliminary trials showed that CFN could be bound to silanized glass surface rendering antimicrobial activity to the glass. In addition, the particles showed antioxidant capacity. **Therefore, it was concluded that the aims of the feasibility study had been successfully reached and that the answer to the “go/no go” question is positive.**

Research outcome. The findings of the feasibility study were/will be presented at two scientific conventions. A paper summarizing the results of the study is currently in preparation. The proposal for a 3-year continuation study has been submitted to BARD. The continuation study will proceed along the following major directions: (a) further enhancement of the particles' activity based on understanding their mode of action; (b) preparing efficient antimicrobial food-contact surfaces and optimizing their performance; (c) testing the efficacy of the antimicrobial surfaces in real food trials. Realization of this program will result in reducing risk of foodborne disease outbreaks and improving quality of food products without overusing synthetic sanitizers and preservatives.

Table of Contents

Cover Page	1
Abstract	3
Table of Contents	4
List of Abbreviations	5
Research problem and its general background	6
Problem 1: Microbial contamination leads to foodborne diseases and spoilage	6
Problem 2: Oxidative spoilage of foods	7
Innovative approach: Active antimicrobial and antioxidant surfaces	7
A new/old generation: phenolic-based active nanomaterials	9
Research objectives	10
Materials and Methods	10
Chemicals	10
Screening MBCD-encapsulated phenolic compounds	11
Preparation and characterization of CFN	13
Testing antibacterial activity of free and glass-bound CFN	13
Spectroscopic measurements	14
Results	15
Active principle selection: curcumin as a promising antimicrobial compound	15
Preparing and characterizing free and glass-bound CFN	17
Antibacterial activity of free and glass-bound CFN	18
Summary and further research directions	19
Relevant bibliography	21

List of Abbreviations

ABTS - 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ATRP - atom transfer radical polymerization
AUC – area under the curve
BHA - butylated hydroxyanisole
BHT - butylated hydroxytoluene
CAC – Codex Alimentarius Committee
CDC - Center for Disease Control
CFN – curcumin-functionalized nanoparticles
DMPC - dimyristoyl-sn-glycero-3-phosphocholine
EDC - 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride
FTIR – Fourier-transform infrared spectroscopy
GC/MS – gas chromatography/mass-spectrometry
IC₅₀ - half maximal inhibitory concentration
LC/MS – liquid chromatography/mass-spectrometry
MBCD – methyl- β -cyclodextrin
MIC - minimum inhibitory concentration
NHS - N-hydroxysucciniamide
NMR – nuclear magnetic resonance
NP – nanoparticles
NYDB - nutrient yeast dextrose broth
PBS - phosphate buffered saline
PDA - polydiacetylene
PDB - potato dextrose broth
PEG - polyethylene glycol
Ph-MBCD – phenolic-MBCD complex
SEM – scanning electron microscopy
SPME - solid-phase microextraction
TEAC – Trolox equivalent antioxidant capacity
TEM – transmission electron microscopy
TLC - thin-layer chromatography
TSA - tryptic soy agar
TSB - tryptic soy broth

Research problem and its general background

Problem 1: Microbial contamination leads to foodborne diseases and spoilage

The Center for Disease Control (CDC, 2010) estimates that there are 76 million foodborne illness cases each year in the USA, leading to 325,000 hospitalizations, 5000 deaths and overall economic loss of \$152 billion. The epidemiology of foodborne diseases in Israel follows the same trends as in the USA (Weinberg and Keller, 2005) and presents a serious healthcare problem (Shvarts, 2009). The most common foodborne infections are caused by *Campylobacter jejuni*, *Salmonella enterica* and *Escherichia coli* O157:H7, but other bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Staphylococcus aureus*, as well as viral and protozoa pathogens may be also transmitted through food consumption (CDC, 2010).

Cross-contamination during food preparation is one of the most common paths of disease spread (Gillespie et al., 2005; Rangel et al., 2005). It typically includes transferring bacteria from contaminated raw materials or infected people via food-contacting surfaces onto food products, and is especially dangerous in the case of ready-to-eat foods. According to a survey by FDA (2004) the hygienic condition of food-contact surfaces in 25% of elementary schools inspected and in 58% of supermarket deli departments did not comply with the regulations.

Cleaning and disinfection of food contact surfaces are required for preventing cross contamination. Disinfection is defined as reduction, by means of chemical agents and/or physical methods, of the number of microorganisms in the environment, to a level that does not compromise food safety or suitability (CAC, 2003). Approved chemical disinfectants for food-contact surfaces are chlorine, iodine and quaternary ammonium. New methods include application of ozone, peracetic acid and electrolyzed water (Fraser and Pascall, 2010). However, most disinfectants decompose in contact with organic matter demanding frequent repeated application. They taint the food and pollute the environment with toxic residues and byproducts and lack acceptance by the public.

In addition, pathogenic and spoilage microorganisms in prepared foods are controlled where permitted by applying physical means (thermal pasteurization, irradiation) and/or adding antimicrobial preservatives such as sorbate and benzoate derivatives. However, these interventions may alter food quality and leave residues not favored by consumers. The industry is seeking for natural substitutes for chemical preservatives and disinfectants. Therefore, there is a need for efficient and consumer-friendly means to keep the contact surfaces and food products free from hazardous microbial contamination without applying conventional chemical, thermal or radiation treatments.

Problem 2: Oxidative spoilage of foods

In addition to microbial spoilage, oxidative reactions are another important cause of food deterioration. Oxidation of unsaturated lipids not only produces offensive rancid odors and flavors but can also decrease the nutritional quality and safety by the formation of potentially harmful secondary reaction products (Frankel, 1980). Lipid peroxidation products and their derivatives are involved in initiation and promotion of chronic diseases, such as cardiovascular diseases and cancer (Ames et al., 1993). Enzymatic browning of mechanically damaged fruit and vegetables is another example of oxidative deterioration of food products. Noteworthy, this discoloration is aggravated in the presence of bacterial contamination (Richard et al., 2009).

The rate of food oxidative degradation depends on oxygen permeability of packaging materials. For example, citrus juices deteriorate much faster in modern plastic or laminated carton packages than in traditional glass bottles or jars (Miltz et al., 1990; Berlinet et al., 2007). The deterioration includes vitamin C degradation, discoloration, flavor and aroma changes. Inhibition of oxidative reactions by improving package barrier properties, using oxygen scavenger (Ros-Chumillas et al., 2007) or adding antioxidant preservatives like sodium metabisulfite or ascorbic acid (Roig et al., 1994) extended the juice shelf life. However, sodium metabisulfite has potential negative health effects (Atkinson et al., 1993), while ascorbic acid may enhance non-enzymatic browning of the juice (Roig et al., 1999).

Other antioxidant food additives, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) also have controversial health reputation. Although their previous allegations in carcinogenicity have been disproved (Williams et al., 1999) they do have several negative health effects (Lanigan and Yamarik, 2002) justifying the consumers' reluctance towards these synthetic chemicals. Extracts of some aromatic herbs such as rosemary or oregano are potent natural antioxidants capable to control food oxidation (Yanishlieva et al., 2006). However, their implementation is limited due to the distinct foreign taste and odor imparted to foods.

Therefore, there is a need for a method to simultaneously control oxidative deterioration and microbial contamination of food products without their tainting with potentially hazardous chemicals and without affecting the organoleptic and nutritional quality.

Innovative Approach: Active antimicrobial and antioxidant surfaces

The concept of antimicrobial surfaces capable of inhibiting microbial pathogens and spoilage organisms by direct contact is a novel approach. It is assumed that such surface will not

require use of conventional chemical, thermal or radiation sanitizers and keep itself free from microbial growth.

Several antimicrobial surfaces have been developed to inhibit the growth of organisms on chronically moist surfaces such as those often found in food industry workplaces (Quintavalla and Vicini, 2002). Most release an active ingredient from the surface that interacts with microorganisms on the surface either to inhibit reproduction or to kill the organism. Other strategies bind the active ingredient to the surface and kill only on contact (Madkour *et al.*, 2009). Eluting antimicrobial surfaces can utilize organic or inorganic antimicrobials. Commonly used organic antimicrobials include triclosan, tri butyl tin, oxybisphenoxarsine and zinc pyrithione (Feied, 2004). Such substances can be used effectively in many settings, but do not hold up well under high-wear or long-life applications and are relatively unstable under the high temperatures used in food manufacturing.

The most promising of the inorganic antimicrobials are the metals silver, copper and zinc, along with oxidative agents such as peroxides and reactive oxygen generators. Among these metals, silver is the least cytotoxic. Although silver is recognized as an effective antimicrobial, effective deployment requires the proper ionic species at the right concentration. Most silver compounds have inadequate solubility and stability in the chemistry of coatings. Traditional elution chemistries are difficult to control, being strongly affected by temperature, flow, pH and the local chemical environment (Feied, 2004).

The use of nanotechnology for creating antimicrobial surfaces for consumer applications is a very active area. There are several ongoing researches; however we are briefly reviewing the previous works that try to probe the mechanisms of killing human pathogens. Tiller *et al.* (2001) used poly(4-vinyl-N-alkylpyridinium bromide) covalently attached to glass surfaces to kill airborne bacteria on contact. The designed surfaces effectively killed 99.9% of *Staphylococcus epidermidis*, *E. coli* and *Pseudomonas aeruginosa*, but only 94% of *S. aureus*. Higher polymer length had higher contact killing rate in case of all bacteria tested. Lin *et al.* (2002) demonstrated that another family of N-alkylated polyethylenimines was equally lethal to bacteria. They tested immobilized polycations on nanoparticles and glass slide surfaces. Structure-activity relationship analysis revealed that in order to create bactericidal surfaces immobilized long polymeric chains have to be hydrophobic and positively charged. Kurt *et al.* (2007) reported design of contact antimicrobial surface via polymer surface modifiers using alkylammonium polyurathanes as antimicrobials. The designed contact surface killed Gram-negative *E. coli* and Gram-positive *S. aureus* by 4.4 and 3.6 log reductions, respectively. Madkour *et al.* (2009) designed fast disinfecting

antimicrobial surfaces and tested them against *E.coli* and *S. aureus*. They functionalized silicon wafers and glass surfaces with facially amphiphilic antimicrobial copolymers using the “grafting from” technique. Surface initiated atom transfer radical polymerization (ATRP) was used to grow poly(butylmethacrylate)-co-poly(Boc-aminoethyl methacrylate) from the surface. Surfaces with varying grafting density and polymer thickness from 2 to 70 nm were prepared. The killing ability of the polymer was independent of polymer layer thickness and density. They could not prove any mechanism of kill and speculated that the death might be due to release of calcium ions from the bacterial phospholipid membranes.

Another promising direction of nanotechnology is development of antioxidant nanoparticles. In particular, antioxidant health-promoting properties were discovered in cerium oxide and yttrium oxide nanoparticles (Schubert et al., 2006).

However, the drawback of the active nanoparticles presented in this section is that they are based on exotic chemicals rather than on natural food-grade substances. This drawback limits the applicability of these approaches in food industry.

Active antimicrobial and antioxidant surfaces and particles based on natural materials are required for food applications.

A new/old generation: phenolic-based active nanomaterials

Many natural plant extracts combine antimicrobial and antioxidant properties (see, for example, Samec et al., 2009; Ksouri et al., 2009). Natural phenolic compounds are usually responsible for this combination of antimicrobial and antioxidant activity. Their additional advantages are being nontoxic, environment friendly and biodegradable. Among the natural compounds combining antimicrobial, antioxidant and health-promoting properties, are well known phenolic compounds, such as resveratrol from grapes (Filip et al., 2003), curcumin from turmeric (Parvathi et al., 2009), hydroxytyrosol from olive (Furneri et al., 2004). Different hypothesis has been proposed for the mechanisms of bacterial inhibition by polyphenols, including inactivation of microbial proteins, disruption of membrane lipid bilayer, or cation binding. Interestingly, phenolic materials perform as a kind of active antimicrobial surfaces in nature. In response to pathogen attack fast biosynthesis of phenolic compounds impregnates the cell wall with antimicrobial phenylpropanoids such as *p*-coumaric, ferulic, and sinapic acids and other lignin-related materials and turns the cell wall into antimicrobial surface similar to those discussed in the previous section (Ascensao and Dubery, 2003). Phenolic components of lignin such as hydroxylated and methoxylated aromatic acids and aldehydes have antimicrobial and antioxidant properties (Barber et al.,

2000; Cruz et al., 2001).

Using natural phenolic compounds as a basis for active food-contact surfaces is still in its infancy. Rhim and Ng. (2007) proposed to use nanocomposite technology to produce environmentally friendly antimicrobial food packaging and food-contact surfaces by blending nanolignin with biodegradable polymers. This approach proved feasible in textile industry when fabrics with antimicrobial activity were produced by adding lignin nanoparticles to the fiber (Zimniewska et al., 2008) or treating wool fabric with curcumin (Han, Yang, 2005). The very recent work reports that immobilization of caffeic acid on polypropylene film renders it antioxidant activity (Arrua et al., 2010).

However, there are almost no works trying to develop active surfaces/nanomaterials possessing both antimicrobial and antioxidant properties simultaneously. Our proposed work has been one of the first attempts in this direction.

Research objectives

The ultimate goal of this research was improving safety and quality of foods by developing novel antimicrobial and antioxidant food-contacting materials using active phenolic-bound nanoparticles. The 1-year study aimed at testing the working hypothesis that at least some properly selected phenolic molecules retain their antimicrobial and antioxidant properties when grafted on liposome-type nanoparticles. In order to reach this immediate aim, the work comprised the following major steps:

- selecting the most promising phenolic compounds,
- building nanoparticles with selected phenolics grafted on their surface,
- testing antimicrobial and antioxidant properties of these particles

Materials and Methods

Chemicals

The phenolic compounds p-coumaric acid, caffeic acid, ferulic acid, sinapic acid, coniferaldehyde, sinapaldehyde, coniferyl alcohol, sinapyl alcohol, hydroxytyrosol, resveratrol and curcumin, as well as methyl- β -cyclodextrin (MBCD, mol. weight 1331) were supplied by Sigma-Aldrich Israel (Rehovot, Israel). Methylene chloride, diethyl ether, chloroform, magnesium sulfate, N-hydroxysuccinimide (NHS) and 1-(3-(dimethylamino) propyl)-3-ethylcarbodiimide hydrochloride (EDC) were from Fisher Scientific, Fair Lawn, NJ, 10,12-pentacosadiynoic acid from GFS Chemicals, Columbus OH, dimyristoyl-sn-

glycero-3-phosphocholine (DMPC) from Avanti Polar Lipids, Alabaster, AL, and ethanol from Bio-Lab, Jerusalem, Israel. Nutrient media tryptic soy agar (TSA) and tryptic soy broth (TSB), LB broth (Lennox) and potato dextrose agar were purchased from BD Difco Laboratories, Detroit, MI. The nutrient yeast dextrose broth (NYDB) was prepared using nutrient broth (Difco), yeast extract (Pronadisa Conda, Madrid, Spain) and D(+) glucose (Merck, Darmstadt, Germany), 0.8, 0.5 and 1.0 g per 1 L water, respectively.

Screening antimicrobial and antioxidant activity of MBCD-encapsulated phenolic compounds

Microencapsulation of phenolic compounds. The phenolic compounds tested in this study are presented in Fig. 1. Equal volumes of 20 mM ethanolic solution of a phenolic compound tested and of 20 mM aqueous solution of MBCD were mixed and stirred for 2 h on a magnetic stirrer at room temperature. Ethanol was removed from the mixture by evaporation under vacuum to half-volume (Rotavapor R-124, Buchi, Flawil, Switzerland). The non-encapsulated residue of the phenolic compound was removed by filtration through sterile 0.45 μ Millex filter with Durapore membrane (Millipore, Cork, Ireland). The obtained filter-sterilized aqueous dispersion of the phenolic-MBCD complex (Ph-MBCD) was kept in refrigerator until use. The concentration of the phenolic compound in the Ph-MBCD was determined by diluting the aqueous dispersion with ethanol in ratio 1:9 and measuring its absorbance with Ultrospec 2100 pro spectrophotometer (Amersham Biosciences, Piscataway NJ) at a characteristic wavelength in comparison with calibration curve of the compound in 90% ethanol.

Assay of antimicrobial activity of MBCD-encapsulated phenolic compounds. Cells of *Escherichia coli* (ATCC 25922) were grown overnight in LB liquid medium, separated from the medium by centrifugation, washed with sterile water and resuspended in a fresh double-strength LB medium to reach optical density of 0.2 at wavelength 600 nm. The bacterial suspensions were further diluted 1:1 with aqueous Ph-MBCD dispersions and sterile water to obtain various concentrations of phenolics, but the same initial *E. coli* dilution. The bacterial suspensions were aseptically pipetted into the wells of 96-well microplate, 0.2 mL per plate and incubated for 20 h at 27°C in EnSpire multilabel plate reader (Perkin Elmer, Waltham MA, USA) with hourly 10 s shaking. The experiments were run in triplicate and in addition to treatments (viable *E. coli* + Ph-MBCD) included blanks (nutrient medium without *E. coli* + Ph-MBCD), positive controls (viable *E. coli* + nutrient medium and MBCD without phenolics) and negative controls (*E. coli* killed by boiling + Ph-MBCD). The bacterial growth

was registered by hourly measurement of turbidity as optical density at 600 nm and evaluated as added area under the growth curve (AAUC) during the whole cultivation period (20 h). Growth inhibition was calculated as AAUC reduction (%) in the presence of a given phenolic concentration compared with a positive control without phenolics. The half-maximal inhibitory concentration (IC_{50}) was determined as a concentration required for 50% growth inhibition, and minimum inhibitory concentration (MIC) as the lowest concentration resulting in complete growth prevention. Similar technique was used for evaluating the effect of curcumin-MBCD complex on yeast growth, except for using the NYDB instead of the LB medium.

Figure 1. The phenolic compounds tested in this study: 1- p-coumaric acid, 2- caffeic acid, 3- ferulic acid, 4- sinapic acid, 5- coniferaldehyde, 6- sinapaldehyde, 7- coniferyl alcohol, 8 – sinapyl alcohol, 9 – resveratrol, 10 – hydroxytyrosol, 11 – curcumin.

Assay of antioxidant activity of MBCD-encapsulated phenolic compounds.

Antioxidant activity of the MBCD-encapsulated phenolic compounds was evaluated by their scavenging capacity towards stable cation radical $ABTS^{+}$ according to TEAC method in our modification (Vinokur and Rodov, 2006). In addition, the antioxidant activity of the phenolic compounds was evaluated by inhibition of volatile aldehydes accumulation as products of thermal autoxidation of linoleic acid (5 min incubation at 80°C). The SPME GC/MS method was used for headspace aldehydes accumulation.

Preparation and characterization of CFN

Synthesis of PDA-NHS. To a solution of 10,12-pentacosadiynoic acid (0.267 g, 0.713 mmol) in dry methylene chloride (10 mL), NHS (0.0914 g, 0.786 mmol) was added followed by EDC (0.144 g, 0.713 mmol). The solution was stirred at room temperature for 2 h followed by rotary evaporation of the solvent. The residue was extracted with diethyl ether and washed with water three times. The organic layer was dried with magnesium sulfate for 0.5 h and then filtered. The solvent was removed by rotary evaporation to give a white solid powder.

Self-assembly of curcumin-functionalized PDA-NHS nanovesicles. A desired ratio of mixture containing PDA, PDA-NHS and DMPC compounds were dissolved in chloroform in a round-bottom flask and the solvent was then evaporated completely to yield a thin film of monomers. The film was hydrated with curcumin solution in water. The resultant suspension was sonicated at 76 °C for ~15 min using a probe sonicator (Vibronic). The solution was then passed through a 0.8 μ m nylon filter to remove the lipid aggregates and cooled at 4 °C overnight to promote self assembly of the monomers. The final dispersion was optically clear. Free curcumin was removed by dialysis. A Spectra/Por® Biotech Cellulose Ester (CE) membrane (MWCO: 100,000) (Fisher Scientific) was soaked in deionized water for 15 min. The solution was carefully transferred in to the dialysis membrane. The water was changed at 2 h, 8 h, 14 h, 24, and 36 hrs during dialysis. The final solution was collected in a vial covered with aluminum foil.

Preparation of antimicrobial glass surface. To remove all contaminants, glass slides were cleaned by sonication in acetone for 10 min, dried under argon for 5 min, and treated with freshly prepared Piranha solution (70% H₂SO₄, 30% H₂O₂) for 30 min. The glass slides were cleaned with copious amounts of distilled water and dried under vacuum. Silanization was performed by immersing the glass slides in 4% aminosilane solution, 1% acetic acid and 95% ethanol. During silanization container was placed under stirring at room temperature. The glass slides were then cleaned with ethanol for about 10 min. The slides were baked at 110°C overnight and brought back to room temperature. Slides were dipped in liposome curcumin solution for 1hr and then kept in vacuum desiccator overnight. The topology of the particles on glass surface was monitored by atomic force microscopy (AFM), Veeco-TopoMetrix TMX 1010, Santa Clara CA.

Testing antibacterial activity of free and glass-bound CFN

Test cultures preparation (E.coli W1485 and B. cereus). Typical food spoilage microorganisms and common food-borne pathogens used in this study were *E.coli* W1485

and *B. cereus*. Stock cultures of *E. coli* W 1485 and *B. cereus* were obtained from Dr. David P. Clark, Department of Microbiology at Southern Illinois University in Carbondale, Illinois. A colony of test culture (*E. coli* W1485 and *B. cereus*) culture picked with sterile platinum loop. This colony was inoculated in to the 50 ml sterile TSB medium and incubated at 37°C with stirring at 150 rpm for 12 hr.

After incubation period the cells of test culture were harvested by centrifugation (Beckman J2-M1, Schaumburg, IL, USA) at 5500 x g /10 min/ 4 C and the supernatant was discarded. The pellet culture suspended in 50 ml Phosphate buffer saline (PBS) (pH 7.0). Serial dilutions were made and 0.1 ml of each dilution was spread plated onto TSA plates for estimating the cells present in the re suspended pellet culture. TSB was prepared according to the manufacturer's (Difco Laboratories, Detroit, MI) directions.

Antimicrobial activity assay. The antibacterial activity of suspension of nanoparticles with curcumin and food contact material coated with it was studied by shake flask assay method. Two mL of nano-encapsulated curcumin was added in to the sterile conical flask containing 23 mL of PBS. For food contact surface study coated glass slides were placed in a sterile conical flask containing 50 ml of PBS at pH 7.0. One ml of test culture suspension was pipetted in to the flask which brought initial cell density of cell suspension to approximately 10⁵ CFU/ml. Flasks incubated in a gyratory water bath shaker (New Brunswick Scientific Edison, NJ, USA) at 36°C for 48 hr. Viable bacteria in the flasks at different time interval were counted by plating aliquots of serial dilutions onto TSA media and incubating for 18 to 24 hr at 35 C.

Similar approach was used for evaluating the effect of curcumin-containing nanoparticles on the survival of yeast *Saccharomyces cerevisiae* in non-growing medium (PBS at pH 7.4), except for using Nutrient yeast dextrose broth (NYDB) for initial growing of the *S. cerevisiae* culture and Potato dextrose agar amended with 100 ppm chloramphenicol (PDA-A) for plating. The incubation duration was extended to 120 h.

Spectroscopic Measurements

UV-Vis absorption spectra of all of the samples were recorded at room temperature using a Perkin Elmer Lambda 25 (spectral slit width 1 nm) UV/Vis. Spectrometer using a cuvette of 1 mL path length. Fluorescence emission spectra of all of the samples were recorded at room temperature using a Perkin Elmer Lambda 55 (spectral slit width 1 nm) Luminescence luminescence spectrometer. Samples were excited at 370 nm and emission was recorded.

External reflection-absorption Fourier-Transform Infrared (FTIR) spectroscopy was performed on liposomes coated on gold substrates using a Nicolet Nexus 670 FT-IR fitted with nitrogen cooled MCT-B detector and a Velma II variable-angle specular-reflectance accessory operating at a beam incidence angle of 80° with respect to surface normal. The sample measurements were averaged at 1000 scans at 2 cm⁻¹ against a background of bare gold substrate. The spectrometer was purged continuously with dry nitrogen gas to minimize water vapor in the sample.

Results

Active principle selection: curcumin as a promising antimicrobial compound

Promising active principles were chosen from the array of natural plant cell wall defense phenylpropanoids and related phenolic compounds known for their antimicrobial effect. Microencapsulation in methylated β -cyclodextrin (MBCD) was used for testing the activities of the polyphenols in aqueous medium towards *E. coli* as a test organism. Just a few compounds, all having 4-hydroxy-3-methoxy substituents, demonstrated a complete inhibition of *E. coli* growth at concentrations tested: curcumin, coniferaldehyde and ferulic acid (Table 1). Within the series with the same substituents the activity declined in the order aldehyde > acid >> alcohol. **Curcumin showed by far the highest inhibitory effect among the compounds tested and was chosen as the most promising active principle for antimicrobial nanoparticles.** An example of curcumin's effect on *E. coli* is presented in Fig. 1. The MBCD-encapsulated curcumin inhibited also the growth of *Saccharomyces cerevisiae*, although its anti-yeast efficacy was much lower than against *E. coli*.

Figure 2. Effects of curcumin and MBCD concentrations on *E. coli* growth.

Viable or killed by boiling *E. coli* was incubated in LB medium with MBCD-encapsulated curcumin, MBCD alone or without any additives. Note complete growth inhibition at curcumin concentration 0.4 mM and lack of MBCD effect.

Table 1. Inhibitory activity of MBCD-encapsulated phenolic compounds towards *E. coli*

Compound	Group	Substitution	IC ₅₀ mM	MIC mM
p-Coumaric acid	Phenylpropanoid acid	4-hydroxy	9.2	n.d.*
Caffeic acid	Phenylpropanoid acid	3,4-dihydroxy	8.3	> 13
Ferulic acid	Phenylpropanoid acid	4-hydroxy-3-methoxy	4.4	6.4
Sinapic acid	Phenylpropanoid acid	4-hydroxy-3,5-dimethoxy	7.9	n.d.
Coniferaldehyde	Phenylpropanoid aldehyde	4-hydroxy-3-methoxy	3.0	6.8
Sinapaldehyde	Phenylpropanoid aldehyde	4-hydroxy-3,5-dimethoxy	5.6	n.d.
Coniferyl alcohol	Phenylpropanoid alcohol	4-hydroxy-3-methoxy	13.6	n.d.
Sinapyl alcohol	Phenylpropanoid alcohol	4-hydroxy-3,5-dimethoxy	16.0	n.d.
Hydroxytyrosol	Phenethyl alcohol	3,4-dihydroxy	10.5	n.d.
Resveratrol	Stilbenoid	3,5,4'-trihydroxy	6.1	n.d.
Curcumin	Phenylpropanoid dimer	4-hydroxy-3-methoxy	0.17	0.4

*MIC was evaluated only when complete inhibition was reached; n.d. – not determined.

All phenolic compounds tested demonstrated scavenging capacity towards stable free radical cation ABTS^{•+} comparable or exceeding that of the standard antioxidant Trolox (Fig. 3). The similarity of results exhibited by ethanolic solutions and MBCD aqueous dispersions showed that microencapsulation did not interfere with antioxidant effect of the phenolics. In addition, MBCD-encapsulated curcumin was shown to inhibit by 50% the thermally-induced autoxidation of polyunsaturated linoleic fatty acid measured as headspace accumulation of volatile aldehydes by solid-phase microextraction (SPME/GC-MS) method.

Fig. 3. Antioxidant capacity of the phenolic compounds in ethanolic solutions or in MBCD aqueous dispersions towards ABTS^{•+} cation radical.

Preparing and characterizing free and glass-bound CFN

Curcumin-containing liposome-type vesicles having the dimensions in a range of 50 to 400 nm were prepared by self-assembly of bilayers including polydiacetylene (PDA), PDA/N-hydroxysuccinimide (PDA-NHS) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC). Comparing the emission spectra of curcumin in solvents of different dielectric constants with that of the nanovesicle-enclosed curcumin (Fig. 4) indicated that at least part of the curcumin in the nanoparticles was located in relatively non-polar medium (dielectric constant between 4.8 to 7.5) rather than in the aqueous environment. It was suggested that due to its hydrophobicity curcumin molecules tend to migrate from the central liposome cavity to the lipophilic bilayer. Fig. 5 represents the FTIR spectra of the PDA nanovesicles with or without curcumin. The bands at 2849 and 2927 cm⁻¹ correspond to CH₂ stretching (symmetric and asymmetric respectively) of polydiacetylene long carbon side chains.

The PDA/PDA-NHS/DMPC nanovesicles, with or without curcumin, were bound to the surface of silanized glass slides through amidization between the amine groups of the aminosilane and the NHS head groups of PDA-NHS. The AFM picture of CFN on glass surface is presented in Fig. 6.

Figure 4. Emission spectra of curcumin in the solvents of different polarity (upper plate) and emission spectrum of curcumin enclosed in PDA/DMPC nanovesicles (lower plate).

Figure 5. Infrared spectra of cystamine functionalized gold surface before (black curve) and after liposome binding (red curve).

Figure 6. AFM picture of CFN on glass surface.

Antibacterial activity of free and glass-bound CFN

The CFN demonstrated bactericidal activity towards both Gram-negative (*E. coli*) and vegetative cells of Gram-positive (*B.cereus*) bacteria reducing their counts in phosphate buffered saline (PBS) at pH 7.0 from 5 log to undetectable level within 8 h (Fig. 7). Only limited activity (not more than 1 log reduction) was observed against yeast.

Figure 7. Reduction of viable counts of *E. coli* (A) and *Bac. cereus* (B) in PBS at pH 7.0 by CFN (red lines). Blue lines: particles without curcumin (control).

Binding CFN to glass surface rendered antibacterial activity to glass slides. Incubation of bacterial suspensions with curcumin-bound glass resulted in reduction in their counts (Fig. 8). Certain decrease of the bactericidal effect as compared to free particles might be related to the limited contact between the glass-bound curcumin and the suspended bacteria. On the other hand, in the trial with *B. cereus* some antimicrobial effect was observed also with non-functionalized glass-bound particles. The performance of surface-bound nanoparticles demands further investigation.

Figure 8. Reduction of viable counts of *E. coli* (A) and *Bac. cereus* (B) in PBS at pH 7.0 by incubation in the presence of glass slides with CFN (red lines). Blue lines: glass slides with nanoparticles without curcumin (control).

Summary and further research directions

The following major findings and achievements have been reached in course of the study:

- Curcumin was chosen as the most promising phenolic compound due to its high antibacterial activity exceeding other tested compounds by at least one order of magnitude.
- Liposome-type phospholipid/polydiacetylene (PDA) nanoparticles functionalized with curcumin were successfully built.
- It was suggested on the basis of fluorescence spectroscopy that curcumin was bound by hydrophobic forces in the bilayer periphery of the liposomes and therefore might exert a contact effect on microorganisms.
- The curcumin-functionalized nanoparticles (CFN) had strong bactericidal activity towards both Gram-negative (*E. coli*) and Gram-positive (*B. cereus*) bacteria, but only limited effect against yeast.
- In addition to the antimicrobial activity, CFN showed antioxidant capacity.

- CFN were bound to silanized glass surface rendering antimicrobial activity to the glass.
- **Therefore, it was concluded that the aims of the feasibility study had been successfully reached and that the answer to the “go/no go” question is positive.**

The findings were/will be presented at two scientific conventions. Joint publication of the results of the feasibility study:

1. Dogra, N., Makwana, S., Choudhary, R., Haddock, J., Rodov, V. and Kohli, P. **An antimicrobial system for preventing food-borne disease outbreaks.** Poster at PITTCON-2012 convention, Orlando FL, March 2012.
2. Rodov, V., Horev, B., Vinokur, Y., Beno-Mualem, D., Choudhary, R., Makwana, S., Haddock, J., Dogra, N., Kohli, P., and Droby, S. **Antimicrobial and antioxidant activity of phenylpropanoids encapsulated in methylated β -cyclodextrin and in polydiacetylene nanovesicles.** Abstract accepted for publication in the materials of FAV and Health -2012 conference, Dharwad, India, November 2012.

A joint scientific paper summarizing the findings of the feasibility study is currently in preparation and is planned to be submitted to the “Journal of Agricultural and Food Chemistry” in 2012.

The continuation research should convert the results of the feasibility study into solutions applicable in practice. Since the most impressive effects of the CFN were observed in the area of antibacterial activity (compared to antioxidant and anti-yeast effects), it was decided to concentrate further efforts on this aspect, implementing their anti-bacterial potential for reinforcement of food safety and quality by developing active antimicrobial food-contact surfaces. At the same time, understanding mode of action of the antibacterial effect of the nanoparticles is essential for improving their efficacy.

Specifically, the continuation research would pursue the following innovative tasks: (a) further enhancement of the particles' efficacy (developing the “2nd-generation CFN”) based on understanding their mode of action and on photosensitizing properties of curcumin; (b) finding practical solutions for binding the CFN to materials relevant to food technology, e.g. plastic polymers and metals; (c) investigating the effect of curcumin-bound surfaces on bacterial biofilm development and its mode of action; (d) real food trials testing the efficacy of the antimicrobial surfaces for solving food technology problems, such as decontamination of liquid foods against human pathogens and spoilage bacteria without thermal treatments

(“cold pasteurization”), preventing cross-contamination in fresh-cut fruit and vegetable industry, active antimicrobial packaging.

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