Nanofibers as Carrier Systems for Antimicrobial Microemulsions. II. Release Characteristics and Antimicrobial Activity

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ABSTRACT: Antimicrobial nanofibers were prepared by electrospinning microemulsions composed of the essential oil component eugenol solubilized in an aqueous nonionic micellar surfactant solution (Surfynol®465) with poly(vinyl alcohol) (PVA). Nanofibers contained microemulsions composed of 0.75–1.5 wt% eugenol and 5–10 wt % Surfynol. Scanning electron microscopy revealed substantial difference in fiber morphology depending on microemulsion composition with fiber diameters increasing as the concentration of either surfactant or essential oil component in the fibers increased. Release studies suggested a burst release of the encapsulated eugenol, potentially due to the hydrophilicity of the polymeric carrier resulting in rapid dissolution of the carrier matrix and high-fiber porosity. The eugenol release rate depended on the amount of eugenol and surfactant incorporated within the fibers. The antimicrobial activity of nanofibers carrying eugenol was evaluated against two strains of *Salmonella typhimurium* (2476 and 2576) and *Listeria monocytogenes* (Scott A and 101) using a macrobroth dilution assay. Presence of nanofibers in bacterial suspensions successfully suppressed growth of foodborne pathogens and in some cases decreased initial cell numbers. Generally, nanofibers were more efficient against Gram-negative than Gram-positive bacterial strains. Results suggest that addition of microemulsions carrying lipophilic components to polymer solutions subjected to electrospinning offers a novel means to further enhance the functionality of nanofibers. © 2010 Wiley Periodicals, Inc. J Appl Polym Sci 118: 2859–2868, 2010

Key words: electrospinning; microemulsion; Surfynol® 465; eugenol; delivery system

INTRODUCTION

Electrospinning is a technique that is used to fabricate ultrafine fibers with mean diameters ranging from several tens of nanometers to a few micrometers from solutions of synthetic polymers or naturally occurring biopolymers.[1–7] Ultrafine fibers or nanofibers have generated significant interest in a number of industries such as the food, pharmaceutical, personal care, and chemical industries due to their unique mechanical, optical, and thermal properties.[8,9] More recently, electrospun nanofibers have been investigated for their ability to act as novel controlled release vehicles. This may be attributed to the fact that fiber size and thus surface to volume ratio and fiber porosity can be accurately controlled by varying (i) solution composition (polymer type, polymer concentration, solvent type, and presence of cospinning agents), (ii) solution properties (ionic strength, pH, and temperature), and (iii) electrospinning conditions (applied voltage, source-to-target distance, and relative humidity).[10–14]

For example, Kenawy et al. manufactured nanofibers containing ketoprofen, a nonsteroidal, antiinflammatory drug (NSAID), by electrospinning hydrolyzed poly(vinyl alcohol) (PVA) with ketoprofen in water.[15] Yang et al. electrospun raspberry ketone from mixtures of PVA and gelatin in formic acid.[16] Jiang produced core-shell nanofibers that contained BSA or lysozyme in the core by electrospinning aqueous solutions of protein and poly(ethylene glycol) (PEG). These fibers were surrounded by a hydrophobic shell composed of poly(ε-caprolactone) (PCL) that was coaxially electrospun with the hydrophilic core solution using a mixture of DMF and chloroform.[17] Finally, Taepaiboon produced all-trans retinoic acid and α-tocopherol loaded nanofibers from solutions of cellulose acetate in 2 : 1 v/v acetone/dimethylacetamide (DMAc).[18]

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Most of these studies focused on either incorporating water soluble compounds such as, for example, proteins, vitamins, and antioxidants in fibers composed of hydrophilic polymers (e.g., polyethylene oxide (PEO), PVA, gelatin, and dextran) or on inclusion of hydrophobic compounds such as anticancer drugs or lipid-soluble vitamins in fibers composed of hydrophobic polymers (e.g., polylactic acid (PLA), poly(ε-caprolactone) (PCL)). While electrospinning of the former can be conducted in water, short-chain alcohols or acids, the latter typically requires organic solvents to dissolve both the hydrophobic polymer and the target lipophilic compound. The use of organic solvents raise environmental as well as health and safety concerns due to the inherent toxicity of many organic solvents. This is in particular a problem for the food industry, where use of many organic solvents is prohibited. The manufacturing of nanofibers made of hydrophilic polymers containing lipophilic components could thus be of substantial interest to a number of industries, since toxicity and residue concerns could be alleviated.

In Part I of our study, we introduced a new method to produce fibers that are composed of a hydrophilic polymer and a lipophilic compound.[19] Here, nanofibers were prepared by first solubilizing a model lipophilic compound (eugenol, 0.75–1.5 wt %) in aqueous solutions containing surfactant micelles (Surfynol®465, 5–10 wt%) to form eugenol microemulsions. The microemulsions were then mixed with a nonionic synthetic polymer (PVA; M_w = 130 kDa, degree of hydrolysis ≈ 87%) and solutions subjected to electrospinning to induce nanofiber formation. We previously reported that material deposited on the collector plate consisted of nanofibers with a circular cross-section and some surface roughness with mean fiber diameters ranging from 57 to 126 nm depending on the concentration of both the surfactant and the lipophilic antimicrobial. Transmission electron microscopy suggested that microemulsion droplets were homogenously dispersed throughout the nanofibers.

On the basis of these results, we hypothesize that (a) such fibers could rapidly release the encapsulated content due to the migration of antimicrobial microemulsions and the simultaneous dissolution of the matrix polymer and (b) that the release of eugenol-containing microemulsions could render the fibers antimicrobially active. The objective of this part of the study was to test this hypothesis by evaluating the release characteristics of the model antimicrobial lipophilic compound (eugenol) incorporated in electrospun PVA fibers as a function of microemulsion composition and concentration and to determine the antimicrobial activity of fibers containing microemulsions added to a suspension of foodborne pathogens.

MATERIALS AND METHODS

Materials

Chemicals

All solutions were prepared with distilled and deionized water. PVA with a molecular weight of 130 kDa and a degree of hydrolysis of 86.7–88.7 mol %, sodium acetate trihydrate (#71188), and eugenol 99% (4-allyl-2-methoxyphenol) (E51791) were obtained from Sigma-Aldrich (St. Louis, MO). Glacial acetic acid (CAS # 64197, UN 2789) was purchased from Acros Organics (Morris Plains, NJ). Tris HCl (#BP1756–500, pH 7.0) was obtained from Fisher Scientific (Pittsburgh, PA). The nonionic surfactant Surfynol® 465, a surfactant belonging to the class of Gemini surfactants—a special group of surfactants possessing at least two hydrophobic chains and two hydrophilic headgroups, which are adjoined by a short spacer molecule known to be excellent solubilizers[20] was kindly provided by Air Products and Chemical (Allentown, PA). Reagents and polymers were used as received from the manufacturer without further purification.

Microbial test cultures

To test the antimicrobial activity of the produced nanostructures, two strains of *Listeria monocytogenes* (Scott A, 101) and two strains of *Salmonella typhimurium* (2486, 2576) were used. The cultures were obtained from the University of Massachusetts, Department of Food Science culture collection.

Methods

Preparation and characterization of micellar solutions

Surfactant solutions were prepared with distilled and deionized water and reagent grade glacial acetic acid. Bulk surfactant solutions were obtained by dispersing Surfynol®465 in water at room temperature to yield concentrations ranging from 5 to 10% (w/w). Subsequently, eugenol was added to the surfactant solutions at concentrations varying from 0.75 to 1.5% (w/w). These concentrations were chosen based on studies previously conducted in our research laboratory that identified the range of surfactant and eugenol concentrations where microemulsions could be formed.[21–24] The solutions were stirred for approximately 15 min at room temperature until the solutions became optically transparent, which indicated completed solubilization and formation of microemulsions. After sterile filtration (0.22 μm) with a syringe filter (Corning, NY) to remove any impurities, solutions were stored up to 2 weeks at 25 ± 2°C. The *z*-average diameter of microemulsions was measured using a dynamic light scattering technique (Zetasizer Nano, Malvern Instruments, UK).

Polymer—microemulsion solution preparation

PVA solutions were prepared by dissolving 7.5% (w/w) PVA in 1 wt % aqueous acetic acid and heating at 80°C for 3 h to ensure complete dissolution of the polymer. After cooling to room temperature, the polymer solution was blended with eugenol-containing microemulsions to yield surfactant and eugenol concentrations ranging from 5–10% and 0–1.5% (w/w), respectively. After blending of solutions for 2 h to ensure a homogeneous distribution, the polymer-micellar dispersions were immediately electrospun.

Electrospinning apparatus

An electrospinning setup described previously was used to electrospin solutions.[25] Briefly, a predetermined amount of polymer dispersions was placed in a 20-ml glass syringe (Micro-Mate, Popper & Sons, New Hyde Park, NY) with a 0.69 mm diameter stainless steel capillary (Hamilton, NE, No. 91,019) blunt-end tip. The syringe was placed in a syringe pump (Harvard apparatus; 11plus, Holliston, MA), which permitted adjustment and control of solution flow rates. The metal capillary of the syringe was connected to the positive lead of a high-voltage power supply (Gamma High Voltage; ES 30P-5W, Ormond Beach, FL), operated in positive DC mode. A grounded copper plate wrapped with aluminum foil and mounted onto two polypropylene blocks was used as the target collector plate for collection of fibers and/or beads. The target was placed 10 cm from the capillary tip. The syringe pump delivered polymer solution at a controlled flow rate of 0.02 ml/min, while the voltage was maintained at 20 kV and the temperature was controlled at 25°C. These conditions were kept constant throughout all experiments.

Scanning electron microscopy

The morphology of electrospun nanofibers was observed with a field emission scanning electron microscope (FESEM 6320 FXV, JEOL, MA) operated at an accelerating voltage of 5 kV. Nanofibers were electrospun directly onto aluminum SEM stubs, which were mounted on the grounded collector plate. After collection of the fibers, samples were sputter coated with gold in a sputter coater (Cressington 108, Cressington, Watford, UK) for 60 s to reduce electron charging effects.

Eugenol release studies

A previously described method by Yang et al. was adapted to study the release of eugenol from nanofibers.[16] Nanofibers (~ 35 mg) containing microemulsions were submerged directly into 30 mL of the release medium (0.05 mol/L Tris-HCl buffer solution, pH 7) and incubated at 25°C at a rotation speed of 80 rpm. Triplicate samples for each release test were taken from different parts of the same nanofiber membrane. At predetermined intervals ranging from 0 to 300 min, 3 mL of the release solution (supernatant) were removed and replaced by an identical volume of fresh solution. The amount of eugenol carrying micelles released after certain time intervals was quantified by UV–vis spectrophotometry. The absorption maxima were measured at 280 nm (Fig. 1). The amount of eugenol present in the nanofibers was back-calculated from data obtained from a predetermined calibration curve of the essential oil compound. The cumulative release $M_{\text{cumulative}}$ in percent was calculated as:

$$C_{\text{cumulative}} = \sum_{t_0 \to t} \left(\frac{M_t}{M} \right) \times 100(\%)$$

where M_t is the amount of eugenol released at time t, and M is the total amount of eugenol added to the electrospinning solution. Results were reported as means of three measurements. The total amount of eugenol was determined by a method modified from Taepaiboon et al.[26] Released eugenol from nanofibers was of approximately the same concentration as in the electrospinning solution.

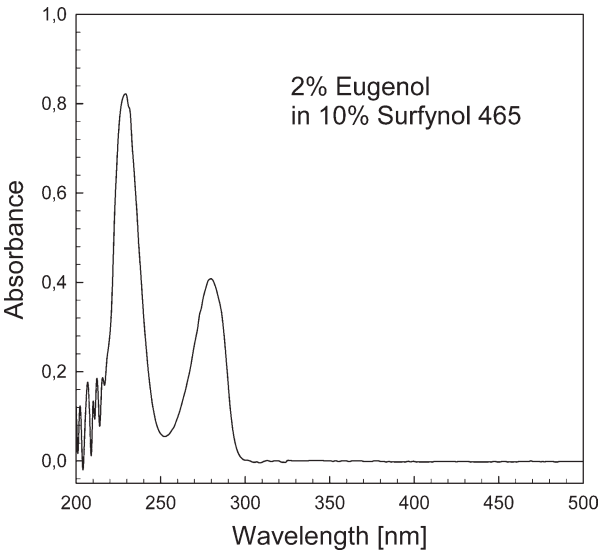

Figure 1 UV–visible absorbance spectra of eugenol microemulsion.

Bacterial cultures

Antimicrobial activity of nanofibers was tested against two different *Listeria monocytogenes* strains (Scott A and 101) and two strains of *Salmonella typhimurium* (2486 and 2576) obtained from the University of Massachusetts, Department of Food Science

culture collection. Bacterial cultures were stored at −75°C in Tryptic Soy Broth with 5% glycerol. Working cultures were maintained on slants stored at 4°C on trypticase soy agar slants (TSA) (Difco Laboratories, Sparks, MD) that were further supplemented with 0.6% yeast extract (TSA-YE) to support growth of *Listeria monocytogenes*. To obtain active cultures for the experiment, a loopful of cells from stock cultures was transferred to Tryptic soy broth (TSB) (Difco Laboratories, Sparks, MD) for *S. typhimurium* and TSB-YE for *Listeria* followed by incubation at 37 and 32°C for 24 h, respectively. Prior to exposure to antimicrobials, each strain was subcultured again in TSB or TSB-YE for 24 h.

Antimicrobial activity assay

The antimicrobial activity of micelle-carrying nanofibers was determined by evaluation of the survival rate after incubation of fibers with pathogens. Controls consisted of PVA nanofibers, PVA nanofibers with Surfynol®465, eugenol microemulsion, and growth controls containing only bacteria and no nanofibers. Samples were comprised of loaded PVA nanofibers electrospun with microemulsions at a surfactant concentration of 10 wt % of PVA and a concentration of eugenol ranging from 0.75–1.5 wt %. Systems containing only nanofibers but no microorganisms were examined for potential bacterial contamination. Test microorganisms at a concentration of 10^5 CFU/mL were inoculated at 25 ± 1°C into each assay system containing growth medium for the bacteria and the nanofibers. Microbial growth was monitoring by enumerating cell numbers in samples after 0, 1, 3, 6, 12, 24, and 36 h of incubation at 25 ± 1°C. Enumeration was carried out by preparing serial dilutions in appropriate concentrations for counting using 0.1% peptone water and the spiral plater (Autoplate®4000, Norwood, MA) for plating of *Salmonella typhimurium* strains on TSA and *Listeria monocytogenes* strains on TSA supplemented with 0.6% of yeast extract. Plates were incubated for 24 h at 32 or 37°C for *Listeria monocytogenes* and *Salmonella typhimurium*, respectively, followed by enumeration using the Q-count system (Q count™, Norwood, MA). Results are means of duplicate experiments.

RESULTS AND DISCUSSION

Microemulsion properties and fiber morphology

Microemulsions composed of eugenol and Surfynol®465 dispersed in 1 wt % acetic acid solutions containing 7.5 wt % PVA had mean particle diameters that ranged from 7 to 23 nm (Fig. 2). The mean particle diameter of microemulsions generally increased with eugenol concentration due to incorporation of the essential oil component eugenol in the palisade layer of the association colloid, but particles remained small enough to prevent light scattering. Solutions were thus transparent at all times. We previously reported in a series of studies on the properties and functionality of these thermodynamically stable antimicrobial association colloids.[21–24] Upon electrospinning of microemulsion-containing PVA solutions, fibers were generated with mean diameters ranging from 57 to 126 nm. Figure 3 shows scanning electron microscopy images of PVA nanofibers containing microemulsions at a Surfynol concentration of 10 wt % and loaded with 0.75–1.5 wt % eugenol at a magnification of 5000 and 10,000. With increasing concentrations of both eugenol and Surfynol, the fibers size increased and fibers increasingly had less bead defects. We previously discussed the underlying reason for these differences in Part I of this study and interested readers are directed there for more in-depth information.[19,27–29]

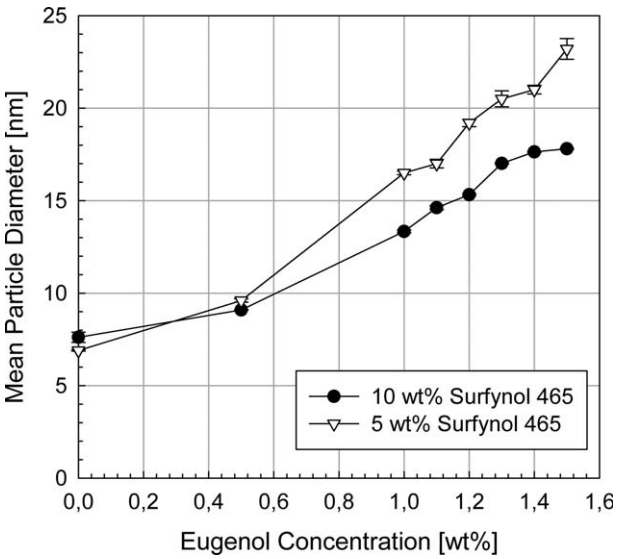

Figure 2 Mean particle diameter of eugenol microemulsions measured by dynamic light scattering as a function of solubilized eugenol concentration at a surfactant concentration of 5 and 10 wt %.

Release of eugenol

Based on the visible differences in fiber morphology shown in Figure 3, functionality of fibers may potentially vary. If nanofibers are going to be useful as antimicrobial delivery systems, then it is critically important that they are able to release the encapsulated eugenol. We therefore measured the rate and extent of eugenol release from selected nanofibers. In particular, we examined the impact of the total amount of eugenol added at either a constant surfactant concentration or at a constant eugenol-to-surfactant loading ratio. Nanofibers were prepared by

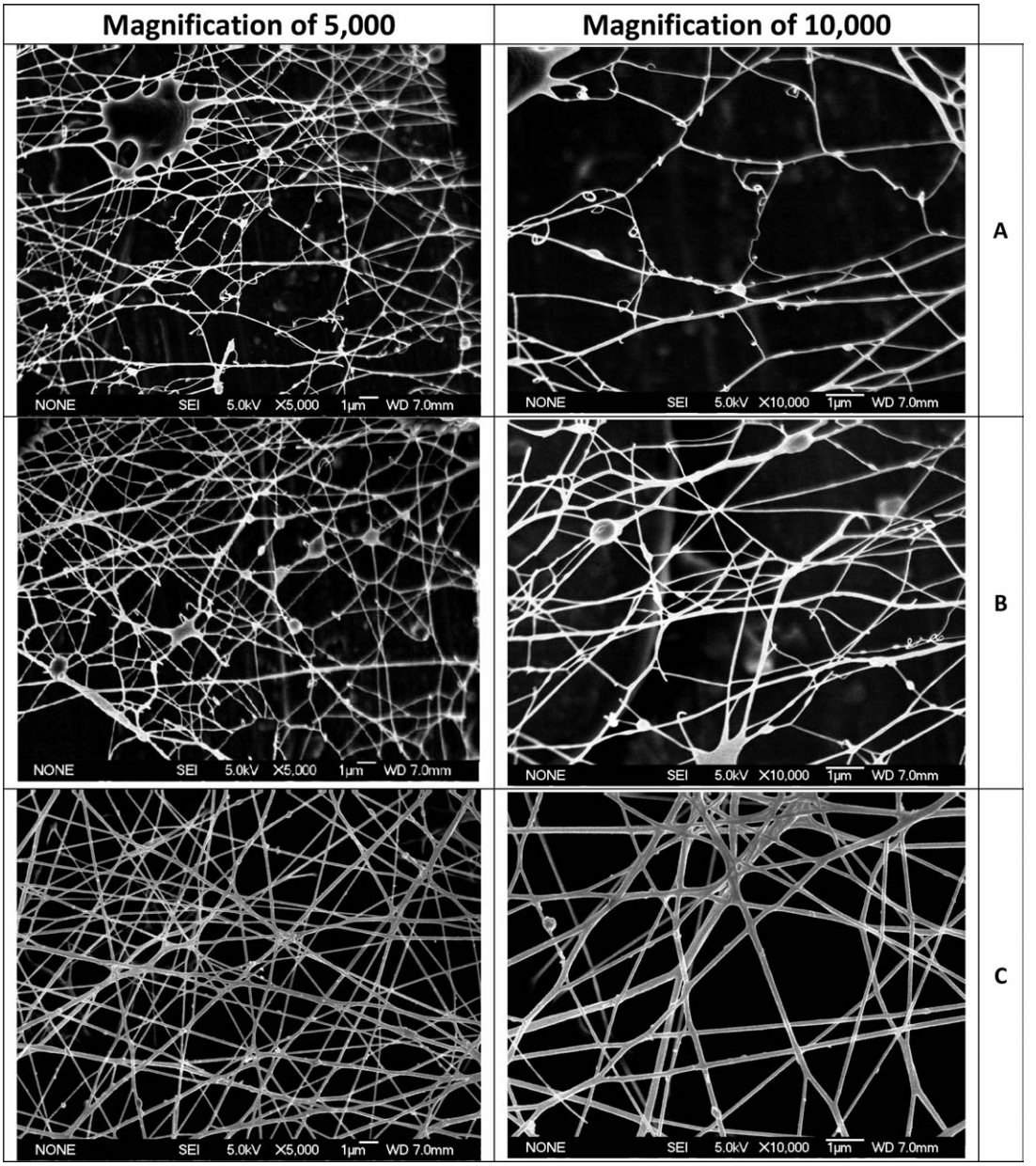

Figure 3 Scanning electron microscopy images of eugenol microemulsion containing PVA nanofibers (7.5 wt %) at a magnification of 5000 and 10,000. Microemulsions contained 10 wt % Surfynol®465 and varying eugenol concentration of (A) 0.75 wt %, (B) 1.125 wt %, and (C) 1.5 wt %.

electrospinning PVA (7.5 wt %), surfactant (5–10 wt%) and eugenol (0.75–1.5 wt%), and then the release kinetics of eugenol from the nanofibers was measured after they were dispersed into buffer solutions (50 m*M* Tris HCl, pH 7) (Figs. 4 and 5).

Initially, we examined nanofiber systems with a constant surfactant concentration (10 wt %), but different eugenol concentrations (0.75 to 1.5 wt %). In all samples, there was a rapid release of eugenol during the first 60 min, followed by a slower release during the next 60 min, after which the amount of eugenol release remained fairly constant (Fig. 4). There are a number of potential physicochemical mechanisms that may contribute to the release of the eugenol into the aqueous solution: (i) initial swelling of the nanofibers in the solution; (ii) the disintegration and dissolution of the nanofibers; (iii) diffusion of eugenol loaded micelles through the nanofibers; and (iv) diffusion of eugenol molecules through the nanofibers. The change in nanofiber structure during the release process was not observed so that we were unable to ascertain the relative importance of these different mechanisms in our study. The initial release rate and the final cumulative amount of eugenol released increased with the total amount of eugenol incorporated into the original system. For

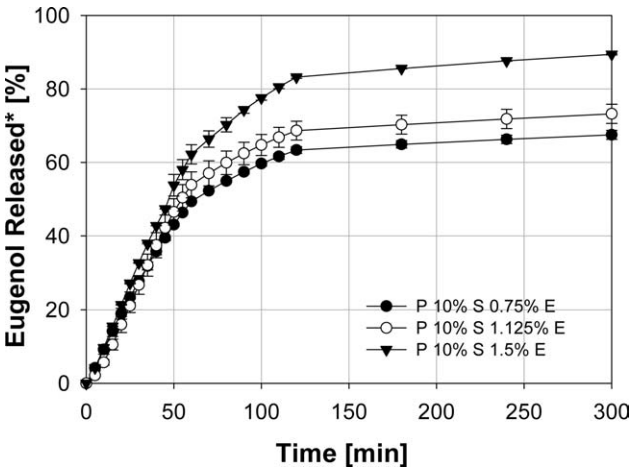

Figure 4 Cumulative release (%) of eugenol from PVA nanofibers containing microemulsion composed of 10 wt % Surfynol® 465 loaded with various amounts of eugenol ranging from 0.75 to 1.5 wt % in 0.05*M* Tris-HCl buffered solution (pH 7). Results are based on the initial eugenol content that was present in nanofibers. *Based on total calculated eugenol content in nanofiber +Letters P, S, and E stand for 7.5 wt % poly (vinyl alcohol), Surfynol® 465, and eugenol, respectively.

example, the amount of eugenol released after 300 min of incubation time was ~ 89%, 73%, and 68% for systems containing 1.5, 1.125, and 0.75 wt % eugenol, respectively. This is in disagreement with the work of Yang et al., who observed that the amount of compounds released increased with decreasing total loading of the compound.[16] One possible reason for the dependence of the rate and extent of compound released on the initial loading could be the relative location of the encapsulated material within the nanofibers. One would expect that material located at the exterior of the fibers would be released more rapidly than material encapsulated within their interiors. Our previous transmission electron microscopy studies suggest that at least some of the eugenol was located at the fiber exterior, perhaps forming a coating around them.[29] In addition, scanning electron microscopy images shown in Figure 4 at the highest resolution indicate a rough and uneven fiber surface, which could be attributed to presence of microemulsion droplets near the surface of fibers. It is possible that only a certain amount of material can be loaded within the interior of the fibers, and once they are saturated the remainder of the material goes to the exterior. Hence, there would be more eugenol present at the exterior of the fibers in systems with high total eugenol loadings, leading to faster release rates. The fact that the majority of the eugenol (>68%) was released from the nanofibers during the 300-min incubation time can be attributed to the large surface area and fine diameter of fibers, as well as the relatively weak attraction between the microemulsions and the fibers in aqueous solution.

In the present experiments, we examined the impact of total eugenol concentration at a constant eugenol-to-surfactant ratio (20 : 3). Previous studies have shown that the loading ratio alters the size of the microemulsions produced, which may contribute to altered diffusion kinetics.[24] For example, incorporation of a higher concentration of eugenol results in an increasing size of the microemulsions, until the micelles have been fully saturated and can no longer take up essential oil components (Fig. 2). To verify that eugenol was released in the surfactant micelles, the UV spectra of pure Surfynol®465 was compared with loaded Surfynol®465 micelles with the result that both showed the characteristic spectra for both compounds (data not shown). Our result is in agreement with the studies by Verreck and Xu, who found that higher loading ratios of an incorporated drug also led to an increase in the absolute amount of drug released.[13,29] Similar release profiles from PVA nanofibers were also observed by others. For example, Zeng et al., who studied the release of BSA from electrospun BSA-PVA nanofibers,[30] observed a fast discharge of the protein from nanofibers. Taepaiboon et al. reported a relatively fast release of four model drugs: sodium salicylate, diclofenac sodium, indomethacin, and naproxen from drug loaded electrospun PVA nanofibers at an incubation temperature of 37°C.[26] Yang et al. reported a fast release of Raspberry ketone from loaded gelatin-PVA nanofibers of varying ratio.[16] In all these cases,

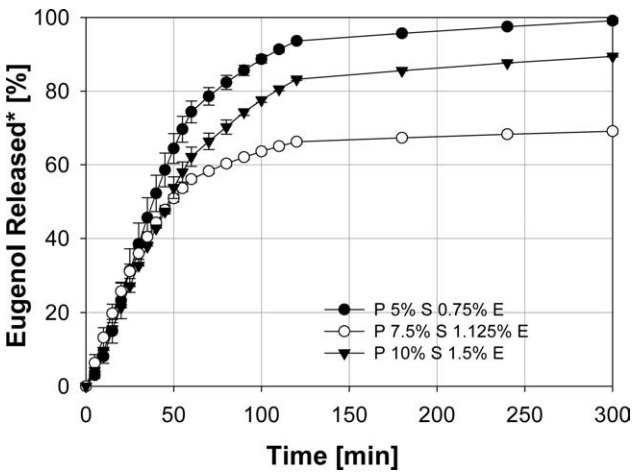

Figure 5 Release profiles of eugenol from PVA nanofibers electrospun with swollen micelles or microemulsion containing 5–10 wt % Surfynol®465 loaded with various amounts of eugenol ranging between 0.75 and 1.5 wt % in 0.05*M* Tris-HCl buffered solution (pH 7). Results are based on the initial eugenol content that was present in each nanofiber. *Based on total calculated eugenol content in nanofiber. +Letters P, S, and E stand for 7.5 wt % poly (vinyl alcohol), Surfynol® 465, and eugenol, respectively.

the partial dissolution of PVA in the reaction medium contributed to the fast liberation mechanism, which, however, also depended on the molecular weight of the compound to be released.

Release profiles of eugenol from nanofibers electrospun from 7.5 wt % PVA solution containing various amounts (5–10 wt%) of Surfynol® 465 with a eugenol loading ranging from 0.75 to 1.5 wt %, respectively, are shown in Figure 5. Interestingly, nanofibers with the lowest total amount of Surfynol®465 and eugenol had the fastest and highest release. However, the release rate at the beginning of the release was similarly high. This initial release leveled off after about 120 min testing time and after 300 min ca. 99% of the total eugenol contained in the specific fiber was liberated into the release medium. The higher concentrated Surfynol®465 of 7.5 wt %, which was loaded with 1.125 wt % eugenol, showed the lowest release percentage of incorporated eugenol. After an incubation period of 5 h, only ~ 70% was discharged from the fibers. In this case, the initial high release rate of samples tested in this set of experiments leveled off after only 50 min, and subsequently only slightly more of the lipophilic compound was released. On the other hand, approximately 89% of eugenol was released from the system containing the highest concentration of both Surfynol®465 and eugenol, namely 10 and 1.5 wt %, respectively. Differences in the observed eugenol content could also be partially due to evaporation effects since the essential oil component exhibits a certain degree of volatility. However, all fiber samples were stored in sealed containers and tested within 1–3 days of fiber production. Some undissolved fiber membrane remained in the sampling system after the completion of the release experiments, which may be attributed to the low-water solubility of PVA at the incubation temperature of 25°C. PVA is fully water soluble at elevated temperatures of ca. 80°C. Similar results were also observed by others.[16,30] To circumvent this and to improve the system for more sophisticated applications, one could potentially crosslink the nanofibers,[15] apply additional coatings[30] or blend with polymers thus incorporating characteristic properties of both polymers,[15] approaches that will be the topic of further investigations.

Antibacterial activity of nanofibers

The ability of eugenol microemulsion-containing nanofibers to prevent growth of food pathogens was tested. Eugenol is known to be a potent antimicrobial against a variety of microorganisms including bacteria and fungi by interacting with their cell membrane.[31,32] Previous studies have shown that the microbial activity of microemulsions is a function of the loading ratio of micelles and the overall concentration of antimicrobial in the system.[24] The antimicrobial efficacy of loaded and unloaded nanofibers and free eugenol microemulsions against two strains of *Salmonella typhimurium* (2486 and 2576) and two strains of *Listeria monocytogenes* (Scott A and 101) as representatives of Gram-negative and Gram-positive bacteria, respectively, is shown in Figure 6(A–D). Concentration of eugenol and Surfynol®465 added as free microemulsion were 500 μg/mL and 5.4 mg/mL, respectively in the test system, while eugenol concentrations in nanofibers was ~ 275, 441, and 702 μg/mL for nanofibers electrospun with 0.75, 1.125, and 1.5 wt % eugenol microemulsion, respectively, at a Surfynol®465 concentration of 53–55 mg.

Antimicrobial activity of nanofibers against *Salmonella typhimurium*

Pure PVA nanofibers and PVA nanofibers containing incorporated Surfynol®465 micelles showed no inhibitory effect on bacterial growth and the test organisms grew at similar levels as the growth controls of both strains grown in TSB in the absence of PVA, surfactant, and eugenol. Addition of free eugenol microemulsion had only a small effect on the reduction or delay of bacterial growth until circa 12 h of incubation time in both *Salmonella* strains where a one log reduction in bacterial number was seen in each case. After 24 h incubation, cells of both strains showed growth at similar levels as the control sample. PVA nanofibers containing eugenol microemulsion showed an initial inhibitory effect for *Salmonella typhimurium* 2486 [Fig. 6(A)] at all three concentrations of eugenol until an incubation time of 6 h, after which bacterial cells rapidly proliferated to the same levels as observed in the control in samples including the lowest amount of eugenol (spun with 0.75 wt % eugenol in the microemulsion), while samples containing a medium amount of eugenol (spun with 1.125 wt % eugenol) initially suppressed growth. Bacterial numbers did not increase for 12 h and increased by 2 log after 24 h. Fibers containing the highest concentration of essential oil component (spun with 1.5 wt % eugenol) showed good antimicrobial activity and initially decreased bacterial cell numbers by 1.5 log within the first 12 h. Cell numbers decreased by 4.5, 3.2, and 2.8 log after 12, 24, and 36 h, respectively, when compared with counts in the growth control.

Electrospun nanofibers containing only 0.75 wt % eugenol in the microemulsion were less effective against growth of *Salmonella* strain 2576 [Fig. 6(B)]. The test organisms grew to levels similar as the control sample, with absolute cell numbers being 0.6–1.2 log lower than counts in the growth control after 12–36 h of incubation. The inhibitory effect of an

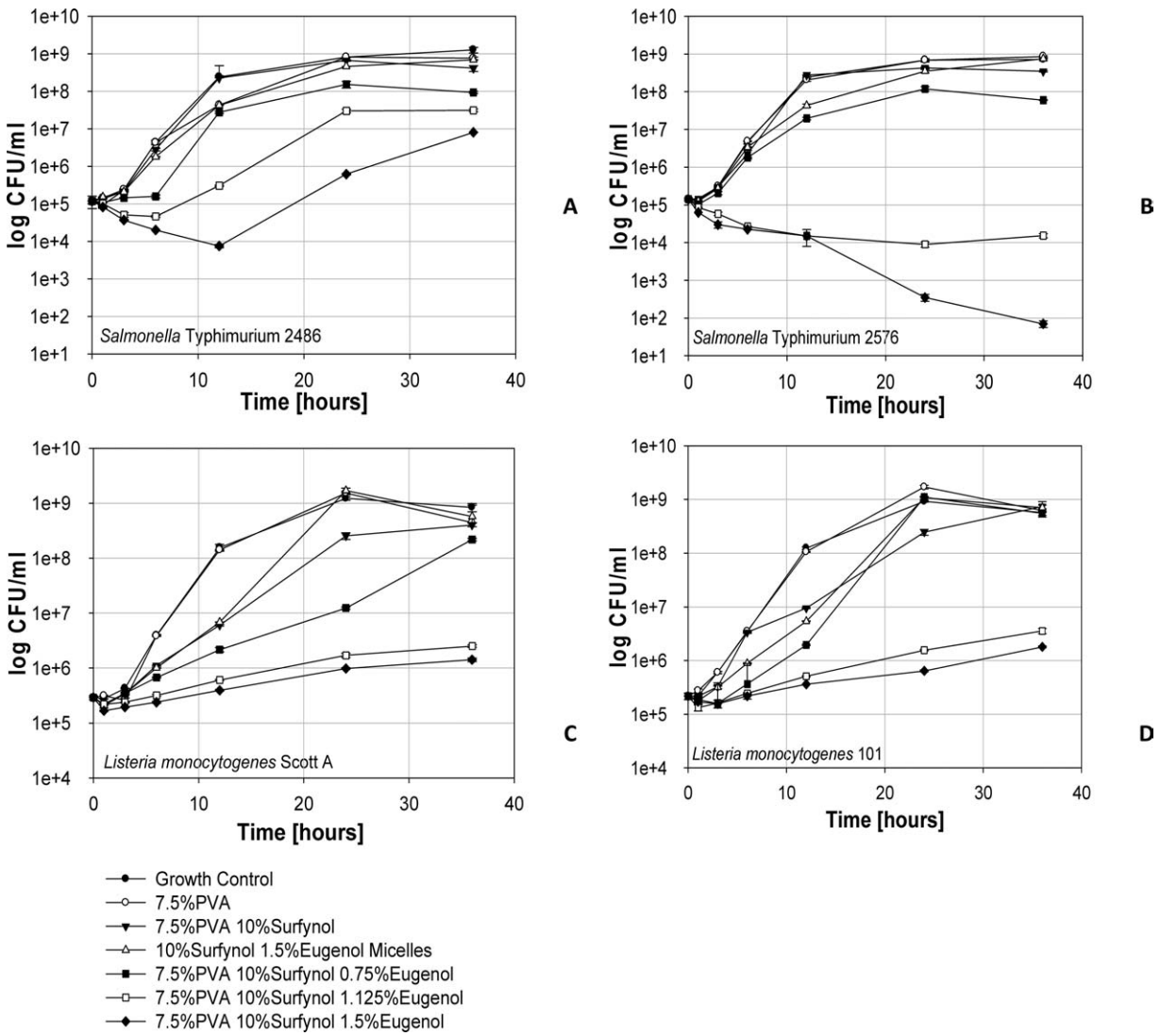

Figure 6 Antimicrobial activity of PVA nanofibers, PVA nanofibers containing 10 wt % Surfynol® 465, PVA nanofibers electrospun with microemulsions of varying loading ratios and pure microemulsion against *Salmonella typhimurium*(A) 2486 and (B) 2576 and against *Listeria monocytogenes* (C) Scott A, and (D)101.

increased eugenol concentration of 1.125–1.5 wt% in micelles electrospun with PVA solution was much more pronounced with strain 2576. For example, initial bacterial counts were reduced by $\sim$ 1 log after 12 h at both concentrations which constitutes a 4.1 log reduction compared with counts in the growth control. After 36 h, initial counts were reduced by 1.3 and 3.5 logs in fibers electrospun from 1.125 and 1.5 wt % eugenol microemulsion in PVA, respectively, corresponding to a reduction of 4.5 and 7 logs compared with counts in the growth control.

Antimicrobial activity of nanofibers against *Listeria monocytogenes*

Both strains of *Listeria monocytogenes* were less sensitive against the action of eugenol when compared with *Salmonella typhimurium*. While no bactericidal activity was observed, bacterial growth was inhibited by PVA nanofibers containing eugenol. For controls, bacterial counts increased by 3.5 logs after 36 h of incubation for both *Listeria* strains. After 12 h of incubation, growth in the presence of eugenol microemulsions was initially lower than in controls [Fig. 6(C)] with cell counts being 1.5 logs lower compared with growth control counts. However, after 36 h, counts were not significantly different from controls indicating that cell grew to similar levels. Nanofibers containing the lowest concentration of eugenol were somewhat more effective against bacterial growth, which is remarkable since the concentration of eugenol in these nanofibers was lower than the eugenol concentration in the free microemulsion (500 μg/mL compared with 275 μg/mL). For example, cell counts increased by 0.9 logs after 12 h with PVA nanofibers containing 0.75 wt % eugenol in microemulsions

compared with initial counts representing a 2 log difference in counts compared with levels in the control. Growth profiles of *Listeria monocytogenes* Scott A in the presence of nanofibers electrospun with higher concentrations of eugenol (1.125 and 1.5 wt %) were very similar. Both showed an increase in bacterial counts of 0.6 and 0.5 log, respectively, after 36 h of incubation time, which represents a $\sim$ 3.8 log reduction compared with counts of the respective growth controls.

Listeria monocytogenes 101 was the most resistant organism to the action of eugenol. The free eugenol microemulsion exhibited some minor activity against this strain with growth being $\sim$ 1 log less than in controls after 12–24 h of incubation. Nanofibers loaded with eugenol microemulsion at the lowest eugenol concentration showed some activity against the bacterium suppressing growth until 12 h by 1.8 log compared with the control [Fig. 6(D)]. However, at longer incubation times of 24–36 h inhibition was lost and microorganisms grew again to levels comparable with the control. Growth profiles of the test organisms containing nanofibers electrospun with higher amounts of eugenol (1.125 and 1.5 wt %, respectively) were again similar. Bacterial growth was completely inhibited for 3 h after which bacterial cell proliferation resumed and gradually increased by 1 or 1.3 log after 36 h, respectively, compared with the initial inoculum level which represents a 2.1 and 2.4 log reduction compared with controls.

Because of its limited water solubility of typically less than 0.01 mol/L,[24] eugenol can be readily incorporated into nonionic surfactant aggregates of Surfynol®465 to form eugenol microemulsion, which substantially increases its solubility. The solubilization capacity depends on the surfactant concentration and more of the essential oil component can be incorporated into the micellar structures as the surfactant concentration increases. Generally, the efficiency of microemulsions has been associated with an increase in eugenol concentration at the interface which directly improves the interaction between the phytophenol and constituents of the bacterial cell wall increasing permeability leading to loss of homeostasis.[33] While antimicrobial efficacy of the microemulsion in our test system was quite low when compared with nanofibers containing a comparable amount of eugenol, it had a slightly inhibiting effect against bacterial growth. However, the concentration of the microemulsion was relatively low when compared with our previous studies that were conducted at approximately 2.25 mg/mL, which is about five times higher than the highest concentration used in this study.[24] The relative higher antimicrobial activity in nanofibers may be attributed to a controlled release mechanism of eugenol from the nanofibers. As shown in the release studies (Figs. 4 and 5) eugenol was continuously and steadily released while in the case of the tested microemulsion, eugenol was already present at a concentration of 500 μg/mL at the beginning of the experiment. This suggests that microemulsions exhausted or lost their activity much more rapidly compared with microemulsions in nanofibers. However, due to hydrophilic nature of PVA, nanofibers potentially lose their structural integrity during the course of the experiments (see previous). In future studies, PVA nanofibers should thus be crosslinked, for example with methanol, or other water insoluble polymers such as poly (lactic acid) may be used, in which case release may however be slowing compared with non cross linked fibers.

Overall, *Listeria monocytogenes* appeared to be more resistant to the activity of eugenol and higher concentrations of eugenol may be necessary to achieve sufficient growth inhibition or kill. This is in agreement with previous studies in our research laboratory that showed a higher antimicrobial efficacy of eugenol against Gram-negative (i.e., *E. coli* and *S. typhimurium*) bacteria.[21] This has been attributed to the composition of outer membrane of Gram-negative bacteria, which contain proteins, lipids, and lipopolysaccharides (LPS) as the major constituents.[34] Surfynol®465 micelles might have a more pronounced interaction with the LPS layer (which does not exist in Gram-positive bacteria) allowing for better interaction of eugenol with the bacterial cell. Furthermore, due to the thinner murein layer, eugenol may subsequently have easier access to the bacterial cell thus allowing for a more effective disruption of membrane functionality, activity of protein bound complexes and maintenance of proton motive forces.

CONCLUSIONS

In summary, functional nanofibers with various morphologies were produced by electrospinning antimicrobial microemulsion carrying poly(vinyl alcohol) blend solutions at varying loading ratios. Release studies revealed an initial burst release in all samples while fibers with a higher loading ratio of eugenol generally liberated more of the compound when compared with fibers loaded at lower loading ratios. Nanofibers exhibited good antimicrobial activity against two strains of *Salmonella typhimurium* and *Listeria monocytogenes*, with inhibition being more pronounced against Gram-negative bacteria cultures. Results of this and the previous study suggest that solubilization of lipophilic compounds in surfactant micelles offers a novel means to generate nanofibers from hydrophilic polymers that contain high concentrations of the lipophilic. Fibers could thereby carry key functional ingredients such as pharmaceuticals, nutraceuticals, flavors,

antioxidants, antimicrobials, or colors, which could greatly widen the range of applications in which nanofibers are currently used.

References

1. Greiner, A.; Wendorff, J. H. Angew Chem 2007, 46, 5670.
2. Ramakrishana, S.; Fujihara, K.; Teo, W. E.; Lim, T. C.; Zuwei, M. 2. An Introduction to Electrospinning and Nanofibers; World Scientific Publishing Co., Pvt. Ltd. 2005.
3. Ramakrishna, S.; Fujihara, K.; Ganesh, V. K.; Teo, W. E.; Lim, T. C. In Functional Nanomaterials; Geckler, K. E., Rosenberg, E., Eds.; 2006; Chapter 7.
4. Ramakrishna, S.; Fujihara, K.; Teo, W. E.; Yong, T.; Ma, Z.; Ramaseshan, R. Mater Today 2006, 9, 40.
5. Ramaseshan, R.; Sundarrajan, S.; Ying-Jun, L.; Barhate, R. S.; Lala, N. L.; Ramakrishana, S. Nanotechnology 2006, 17, 2947.
6. Subbiah, T.; Bhat, G. S.; Tock, R. W.; Pararneswaran, S.; Ramkumar, S. S. J Appl Polym Sci 2005, 96, 557.
7. Supaphol, P.; Chuangchote, S. J Appl Polym Sci 2008, 108, 969.
8. Kriegel, C.; Arecchi, A.; Kit, K.; McClements, J.; Weiss, J. Crit Rev Food Sci Nutr 2008, 48, 775.
9. Weiss, J.; McClements, J.; Takhistov, P. Food Aust 2007, 59, 274.
10. Chew, S. Y.; Wen, J.; Yim, E. K. F.; Leong, K. W. Biomacromolecules 2005, 6, 2017.
11. Jiang, H.; Hu, Y.; Zhao, P.; Li, Y.; Zhu, K. J Biomed Mater Res B: Appl Biomater 2006, 79, 50–57.
12. Suwantong, O.; Opanasopit, P.; Ruktanonchal, U.; Supaphol, P. Polymer 2007, 48, 7546.
13. Verreck, G.; Chun, I.; Rosenblatt, J.; Peeters, J.; van Dijck, A.; Mensch, J.; Noppe, M.; Brewster, M. E. J Controlled Release 2003, 92, 349.
14. Zeng, J.; Xu, X.; Chen, X.; Liang, Q.; Bian, X.; Yang, L.; Jing, X. J Controlled Release 2003, 92, 227.
15. Kenawy, E. R.; Abdel-Hay, F. I.; El-Newehy, M. H.; Wnek, G. E. Mater Chem Phys A 2007, 459, 390.
16. Yang, D.; Li, Y.; Nie, J. Carbohydr Polym 2007, 69, 538.
17. Jiang, H.; Hu, Y.; Li, Y.; Zhao, P.; Zhu, K.; Chen, W. J Controlled Release 2005, 108, 237.
18. Taepaiboon, P.; Rungsardthong, U.; Supaphol, P. Eur J Pharm Biopharm 2007, 67, 387.
19. Kriegel, C.; Kit, K.; McClements, D. J.; Weiss, J. Langmuir 2009, 25, 1154.
20. Menger, F. M.; Keiper, J. S. Angew Chem 2000, 39, 1907.
21. Gaysinksy, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J. J Food Prot 2005, 68, 2559.
22. Gaysinksy, S.; Davidson, P. M.; Bruce, B. D.; Weiss, J. J Food Prot 2005, 68, 1359.
23. Gaysinsky, S.; Davidson, P. M.; McClements, D. J.; Weiss, J. Food Biophys 2008, 3, 54.
24. Gaysinsky, S.; Taylor, T. M.; Davidson, P. M.; Bruce, B. D.; Weiss, J. J Food Prot 2007, 70, 2631.
25. Wongsasulak, S.; Kit, K. M.; McClements, D. J.; Yoovidhya, T.; Weiss, J. Polymer 2007, 48, 448.
26. Taepaiboon, P.; Rungsardthong, U.; Supaphol, P. Nanotechnology 2006, 17, 2317.
27. Kriegel, C.; Kit, K.; McClements, D. J.; Weiss, J. Polymer 2009, 50, 189.
28. Kriegel, C.; Kit, K.; McClements, D. J.; Weiss, J. Food Biophys 2009, 4, 213.
29. Xu, X. L.; Yang, L. X.; Xu, X. Y.; Wang, X.; Chen, X. S.; Liang, Q. Z.; Zeng, J.; Jing, X. B. J Controlled Release 2005, 108, 33.
30. Zeng, J.; Aigner, A.; Czubayko, F.; Kissel, T.; Wendorff, J. H.; Greiner, A. Biomacromolecules 2005, 6, 1484.
31. Dorman, H. J. D.; Deans, S. G. J Appl Microbiol 2000, 88, 308.
32. Walsh, S. E.; Maillard, J. Y.; Russell, A. D.; Catrenich, C. E.; Charbonneau, D. L.; Bartolo, R. G. J Appl Microbiol 2003, 94, 240.
33. Blaszyk, M.; Holley, R. A. Int J Food Microbiol 1998, 39, 175.
34. Kayser, F. H.; Bienz, K. A.; Eckert, J.; Zinkernagel, R. M. Medizinische Mikrobiologie; Georg Thieme Verlag: Stuttgart, 1997.