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A Concept in Bactericidal Materials: The Entrapment of Chlorhexidine within Silver

By *Racheli Ben-Knaz, Rami Pedahzur,* and *David Avnir**

A new concept in bactericidal agents is described: the entrapment of an organic biocidal agent within a bactericidal metal, which leads to synergism between the two components. Specifically this concept is demonstrated for the entrapment of chlorhexidine digluconate (CHD) within an aggregated silver matrix, a metal known for its own biocidal qualities, forming the CHD@silver composite. The bactericidal efficacy against *E. coli* is evaluated and compared with the separate components. While the bactericidal efficacy of the individual ingredients (CHD and metallic silver) is very low, CHD@silver exhibits a markedly enhanced efficacy. This enhanced bactericidal effect is partially attributed to the simultaneous release and presence of the active biocidal ingredients CHD and Ag^+ in the solution. Detailed composite characterization is provided.

1. Introduction

Combinations of antimicrobial agents which exhibit an enhanced biocidal effect are considered beneficial.[1] In addition to their higher biocidal action, they can broaden the biocidal range, reduce the probability of developing resistance, minimize deleterious host side-effects, and reduce the risk of environmental pollution. The development of new combinations of known and registered biocides is even more desirable, because it lowers the complications associated with the development of new biocidal products. Here we report on harnessing a new material technology—the doping of metals with organic molecules[2–4]—as a new concept for the preparation of such combinations; and in particular on the preparation of silver doped with chlorhexidine digluconate, CHD@Ag, and its biocidal activity, which by far surpasses the activity of each component separately. To the best of our knowledge, the doping of an antibacterial metal with an antibacterial organic compound has not been reported to date.

The biocidal activity of metallic silver has been known since the Greek era.[5] Nowadays silver and its compounds are commonly used in many domains: In the clinical area, silver impregnated dressings are used in wound management, particularly in burns and in chronic and diabetic ulcers,[6] and in catheters and other implantable medical devices.[7] Non-clinical applications include its use in water purification processes (such as the Katadyn process[8]), for impregnation of carbon filters,[9,10] in coated domestic appliances,[11,12] in swimming pool electrochemical sanitation,[13] in textiles,[14] as food preservatives,[9,15] in cosmetic preparations,[16] and as nano-silver in computer components.[12]

The biocidal activity of metallic silver is associated with its potency at very low concentrations, defined by Carl Nageli as oligodynamic:[17–19] bulk silver releases trace amounts of silver cations from its surface, which are strongly toxic to a wide range of microorganisms including gram negative and gram positive bacteria, fungi, and even viruses.[20–22] The proposed mechanism for its biocidal activity is closely related to the strong interactions of silver ions with thiol groups in enzymes and in other vital proteins which lead to their inactivation. Thus, exposure to silver ions damages multiple components of bacterial cell metabolism, including the permeability of the cell membrane, which leads to gross cellular structural changes, blockage of transport processes, and interference in the activity of vital enzymatic systems such as the respiratory cytochromes, alteration of proteins, and binding to DNA and RNA, which in turn affects their functionality.[6,17]

Whereas combinations of *cationic* silver with other biocides such as silver sulfadiazine with or without chlorhexidine digluconate,[23] copper,[24] and hydrogen peroxide[25] are well known and used,[9,11] the use of *metallic* silver in biocidal combinations has been limited to the coating or impregnating of biocidal polymers with silver[26] or to the mixing of antibiotics with silver.[27] All of these combinations displayed synergistic activity.

The doping of silver employs, as mentioned above, a recent and general methodology for entrapping organic molecules and polymers within metals, which has already found various useful applications in catalysis[28–30] and bio-catalysis,[31] in alteration of metallic properties such as their reactivity,[2] in the induction of new and not necessarily related properties to metals such as chirality[32] or acidity/basicity,[33] and more.[34] The entrapment process involves a room temperature metal synthesis by chemical reduction of the metal cation, in either aqueous[2] or organic phases,[35] with either a homogenous[2] (solvent-soluble) or heterogeneous[3] (solvent-insoluble) reducing agent, carried out in the presence of the desired organic molecule. Our studies have

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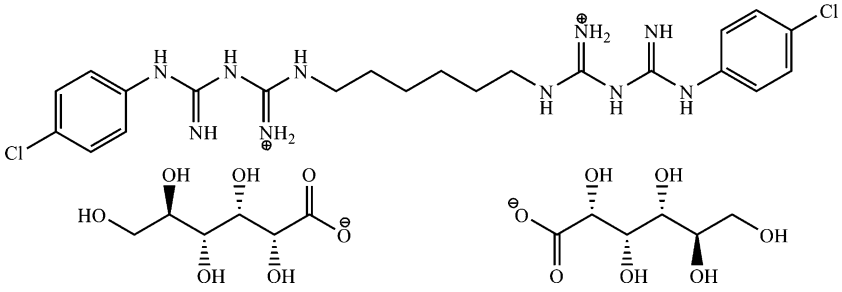

Figure 1. Chlorhexidine digluconate (CHD).

shown that the molecules remain intact upon entrapment, that they reside within closed and narrow pores formed during the growth and aggregation of the metal crystallites, that the metallic matrix is suitable to serve as a carrier for reactive functional molecules, retaining their reactivity, and, that they are accessible for chemical interaction with an external reagent, diffusing into the porous material.[31] It was also shown that the entrapped molecules can be extracted with different solvents[2] and this has led us to propose using the composite as a slow release matrix for organic molecules, as reported here for CHD. CHD (Fig. 1) is a widely used, broad-spectrum antiseptic agent capable of damaging the membranes of both gram positive and negative bacteria. Its entrapment within silver was carried out by using a homogenous methodology, namely using sodium hypophosphite as a reducing agent for the silver cations. The resulting CHD@Ag composite was tested for its bactericidal action against wild type *E. coli*, which is only slightly affected by silver or CHD at the same concentrations and conditions.

2. Results and Discussion

2.1. Material Characterization

A typical high-resolution scanning electron microscopy (HR-SEM) image of the CHD@Ag composite is displayed in Figure 2a. It is seen that the composite powder is made of nanometric metallic crystallites that are aggregated into ~1 μm particles. Coupling

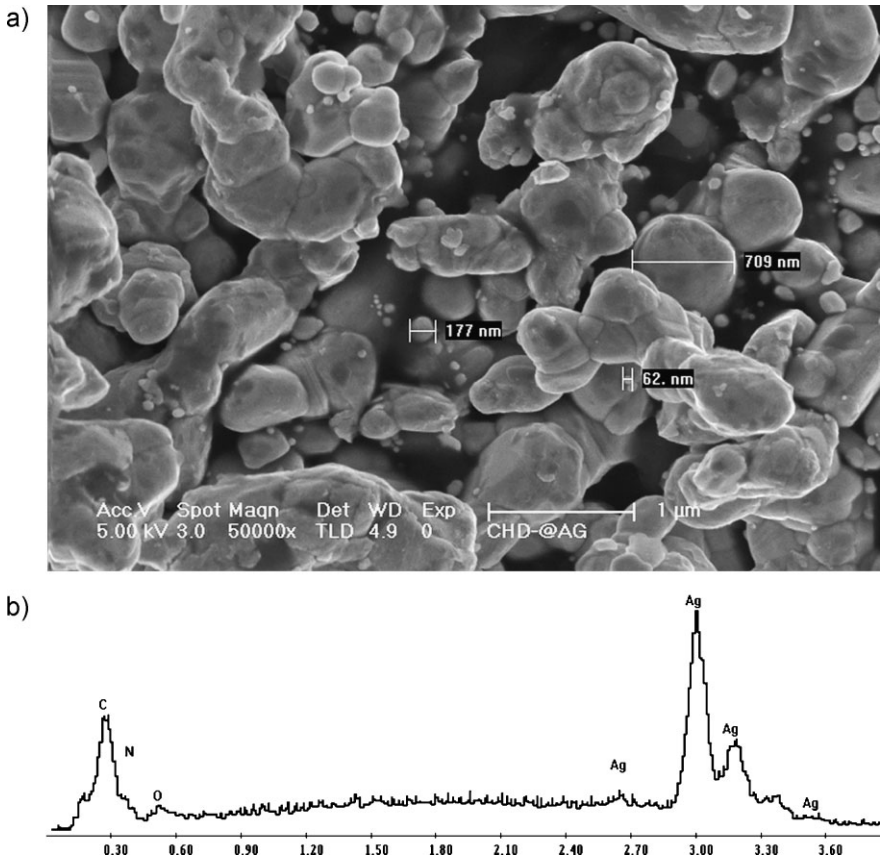

Figure 2. a) HR-SEM image of silver, within which chlorhexidine is entrapped (CHD@Ag). b) EDAX analysis of CHD@Ag.

energy dispersive X-ray (EDAX) analysis (Fig. 2b) with SEM imaging reveals the organic nature of the composite with the appearance of nitrogen, carbon, and oxygen.

In order to examine the chemical stability of the CHD molecules towards the entrapment process, an extraction experiment in MeOH was carried out. Figure 3 compares the spectroscopic analysis of the methanol-extract of CHD@Ag to that of CHD dissolved in MeOH, showing that the CHD molecules remain intact upon entrapment. The extraction experiment also provided the means to determine the amount of CHD present in the composite, which was found to be 0.19% mol of CHD per mol of silver.

The thermogravimetric analysis (TGA) of CHD before and after entrapment within silver is shown in Figure 4. It is seen that the metal catalyzes the oxidative degradation of the entrapped CHD, narrowing the degradation temperature range from 100–700 °C to 200–500 °C, eliminating the ~620 °C degradation step altogether, and shifting the two derivative peaks to lower temperatures. This catalytic effect of the metallic matrix on the oxidative degradation of the entrapped molecules has been observed previously in other studies of organic–metallic composites,[3,33] which indicates the close proximity between the entrapped organic molecules and the metallic pore surface.

The kinetics of CHD leaching from the composite into HEPES buffer (which served as the bactericidal test medium) was measured and the results are presented in Figure 5. It is seen that the release profile of the composite is characterized by a rapid release of about half the entrapped CHD molecules followed by a relatively slow release. This behavior was found to fit the Weibull model:[4,36]

$$m(t) = m_\infty \cdot \left[1 - \exp\left(-\left(\frac{t}{t_c}\right)^b\right)\right] \quad (1)$$

Here, $m(t)$ is the dopant fraction that is released into the extracting solvent at time t, m_∞ is the total fraction that may be extracted by the chosen solvent after infinite time, t_c is a characteristic time, namely the time required for 63.2% (exp(−1)) of the total extractable population be extracted, and b is a shape parameter, which may have values between 0 and 1. If $b = 1$, the model reduces to a simple fist-order model; if $b < 1$, the curve is parabolic, with a higher initial slope (accommodating the initial 'burst' observed in some extractions). The parameter b can thus be taken as an indication of the degree of homogeneity of the extractable population: a value near to 1 implies a relatively homogeneous extractable population with t_c corresponding to its

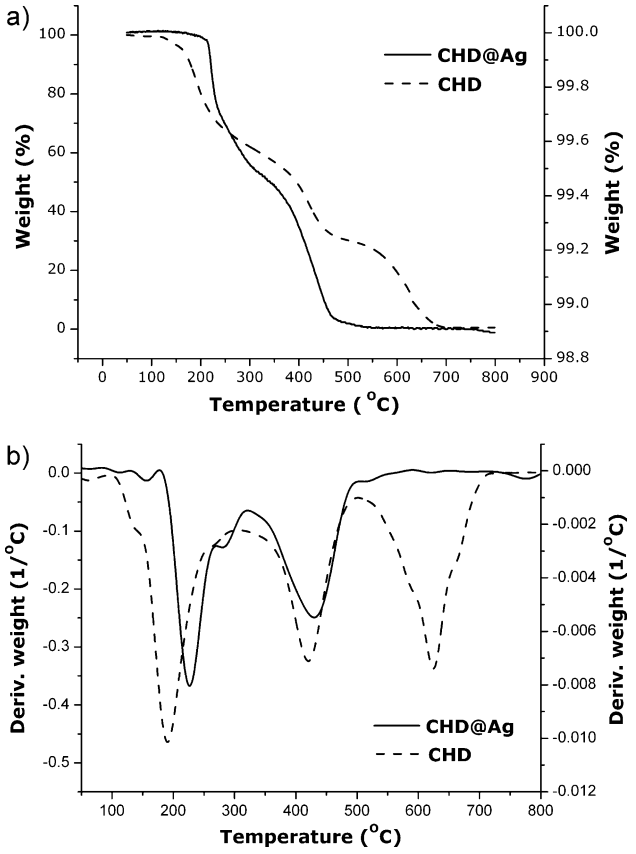

Figure 4. a) TGA in air of pure CHD (left axis) and CHD within CHD@Ag (right axis). b) First derivative of (a).

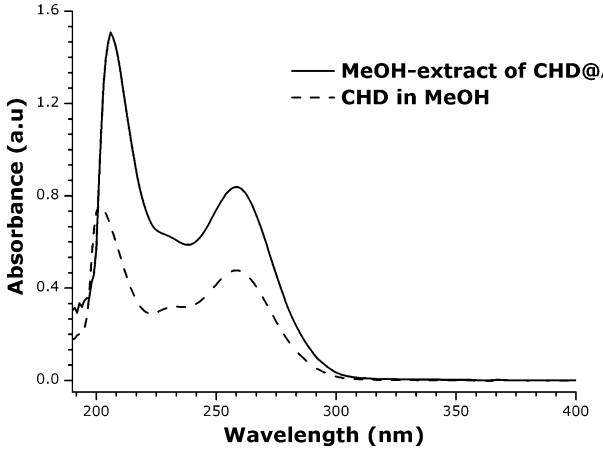

Figure 3. UV spectra of CHD in MeOH before entrapment and after extraction from CHD@Ag.

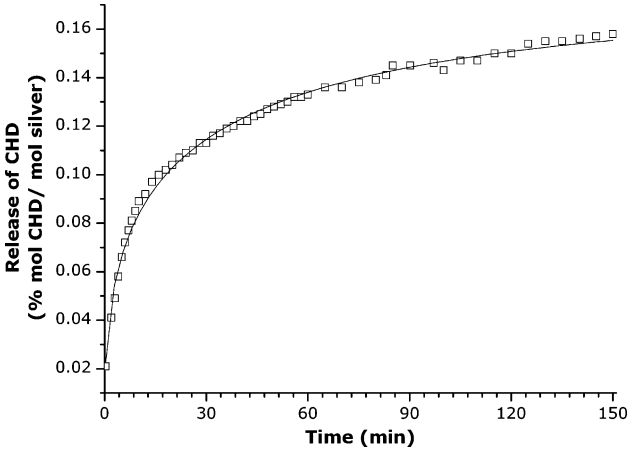

Figure 5. Release profile of CHD from CHD@Ag and its fit to the Weibull model.

first-order characteristic time, while a value far from 1 implies sample heterogeneity. For the curve in Figure 5 the fitting parameters are $t_c = 26$ min and $b = 0.45$, with $m_{t=\infty} = 0.17\%$, $R^2 = 0.99$. This intermediate b value is thus indicative of the two CHD populations: the easily HEPES buffer-extractable accessible population and the slow released CHD. This heterogeneity of the release of CHD is also evident in the shape parameter, $b = 0.45$, which points to the heterogeneity in the released populations. As will be shown later, this release profile dictates the kinetic biocidal profile of the composite. Finally, recalling that the total CHD population of the composite determined by the extraction experiment in MeOH is 0.19%, $m_{t=\infty} = 0.17\%$ implies that about 10% of the entrapped population is held within the matrix much tighter.

It is important to note that the adsorption of CHD on pre-made silver powder and its entrapment within silver are completely different processes. Whereas adsorption takes place on the outer surface of the pre-aggregated metal and is a 2D process, entrapment occurs during the aggregation of the metallic particles into a metallic powder, resulting in a 3D configuration of host and dopant. Thus, while the entrapped molecules are confined within cages, the walls of which are made of the metallic matrix, with hindered access to the solvent, adsorbed molecules are freely facing the solution and thus can easily desorb. Even more striking is the observation that whereas CHD molecules do not tend to adsorb on the metal surface (below the detection limit), entrapment of CHD within the porous network of metallic aggregates occurs as described above. This, in turn means that the entrapment is mainly physical encaging of CHD molecules within the interstitial porosity and cages of the metallic porous network, which allows their release when needed.

2.2. The Biocidal Activity

Figure 6 shows the striking synergetic effect of CHD@Ag towards *E. coli* MG1655. It is seen that this strain is only slightly affected by exposure to silver, that the CHD solution exhibits only very weak biocidal activity, and that the Ag^+ (from $AgNO_3$), to which a role has been attributed in the activity of Ag,[17] shows no biocidal activity. In contrast, CHD@Ag exhibits highly efficient biocidal activity, reducing the population to practically zero (note the logarithmic scale) after 90 min. To make the comparison with silver relevant, the silver used is CHD@Ag from which all of the CHD was extracted.

Interestingly (Fig. 7), the synergetic effect is also observed when either Ag or $AgNO_3$ are pre-mixed with CHD. Whereas the synergetic activity of mixed CHD and Ag^+ is known,[20,23,37–39] we are unaware of previous studies of the elementary mix of metallic Ag and CHD. We thus see that *any* CHD–Ag combination is highly bactericidal. The advantage of CHD@Ag is the longer action, compared with the fast action of the two mixes.

2.3. The Proposed Bactericidal Action

It is well known that combinations of biocidal agents may exert enhanced effects when compared with the sum of their individual ones.[1] While in some cases the chemical or biological rational behind the enhanced biocidal effects is evident, in others it is more elusive. For example, an enhanced combined activity of two agents may stem from their mutual interaction, which may generate active species that are more potent than the original substances. Another option is that one agent may increase the sensitivity of the cell towards the other, for example, by increasing the permeability of the cell towards the second agent hence enhancing its biocidal potential. Still another option is that the two agents may function independently on their specific cellular targets and the cumulative damage may overwhelm the affected cell and be reflected by an enhanced biocidal effect. Of these options, for CHD@Ag and CHD + Ag we believe that the mechanism of action is attributable to the ability of CHD to disrupt bacterial membranes making them more penetrable to the Ag^+ ions which may enter the cell and bind to intracellular moieties such as the DNA and inhibit its replication.[39] The fact that metallic Ag can act as a source for Ag^+ in contact with water through its oligodynamic effect, makes its combination with CHD exhibit the synergistic characteristics of the ionic combination. For the entrapment of an organic biocidal

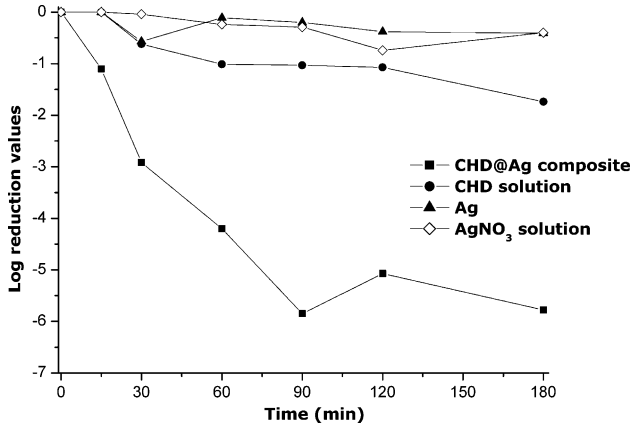

Figure 6. Kinetic profile of the bactericidal activity of the CHD@Ag composite compared with a CHD solution and with silver, towards *E. coli* MG1655.

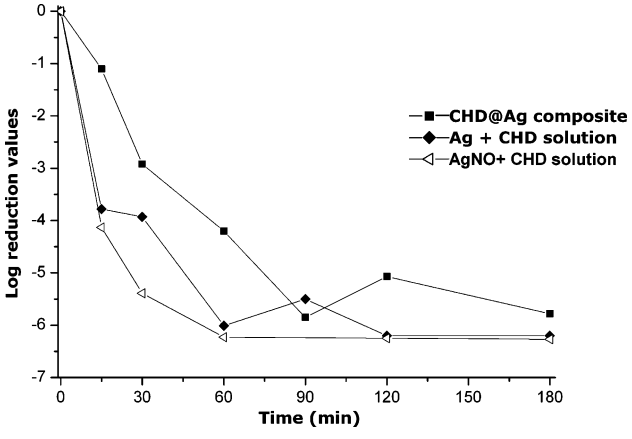

Figure 7. Kinetic profile of the bactericidal activity of the CHD@Ag composite compared with mixed CHD-Ag combinations towards *E. coli* MG1655.

agent within a biocidal metal, which is a new concept in bactericidal agents, the gradual co-release of both CHD and ionic Ag from the metallic matrix is an important feature. As the CHD molecules gradually leave their hosting metallic pores, the extent of the exposed metallic surface increases and thus, the release of Ag^+ increases, and the extended time-release of the composite enables one to achieve longer-lived antibacterial effectiveness. Finally, as noted above, whereas the synergistic biocidal activity of a solution of CHD and Ag^+ is known,[23,37–39] synergism of the straightforward mixture of CHD and silver powder is, to the best of our knowledge, not known; when fast action is needed, this would be the preferred Ag-CHD composite.

3. Conclusions

We have introduced a novel biocidal composite material in which organic biocidal molecules are entrapped within a biocidal metallic porous matrix. It is shown that the entrapped molecules are physically encaged within the pores of the aggregated metal and yet, can be released into the solution and inactivate bacteria synergistically with the natural release of Ag^+ from the metallic surface. In passing we discovered that the highly active simple Ag + CHD mixture is unknown. It is shown that the composite provides a longer biocidal effect, compared with the mix, having a gradual release of the active ingredients.

The concept of entrapping biocidal molecules within a biocidal metal can be further applied to a variety of biocidal agents, including antibiotics, antiviral agents, antifungal agents, bio-film treatment agents, and other metals including copper and gold.[40] We envisage many applications for biocide@metal composites as active ingredients in topical pharmaceutics, in coating of biomedical devices, and in biofilm and water treatment applications.[40]

4. Experimental

Chemicals: $AgNO_3$ was purchased from Metalor. $NaH_2PO_2 \cdot xH_2O$ was purchased from Aldrich (based on data provided from the producer, x in the phosphite was approximately 0.2). A CHD solution, 20% in water, Na_2HPO_4, KH_2PO_4, NaCl, and KCl were purchased from Sigma. Nutrient agar was purchased from DIFCO. Sodium thioglycolate, sodium thiosulfate, lecithin, and HEPES were purchased from Acros Organics. Tween 80 (poly(ethylene glycol) sorbitan monooleate) was purchased from Fluka.

Entrapment Procedure: $AgNO_3$ (3.03 g, 0.018 mol) was dissolved in distilled water (100 mL). CHD solution (0.76 mL of 0.236 M, 0.18 mmol) was added and the combined solution stirred for 2 h at 30 °C. NaH_2PO_2 (1.18 g, 0.013 mol) was then added and the combined slurry was stirred at 30 °C for 4 d. The resulting precipitate was filtered, washed with three portions of distilled water (100 mL each), and dried overnight under vacuum. The resulting composite, 1.9 g of CHD@Ag, contained 0.19% mol of CHD per mol of silver, which is 20% of the biocide initial amount.

Testing for Possible Adsorption of CHD on Silver: To compare adsorption to entrapment, metallic silver was prepared as described above but in the absence of CHD. The resulting powder was stirred for 4 d in a solution of CHD under the conditions and the concentrations of the entrapment experiment. The resulting solid was analyzed by TGA and did not exhibit any weight loss that could be attributed to CHD.

Extraction and CHD Release Profile Experiments: I. Extraction in MeOH: The CHD@Ag composite (0.1 g) was suspended and stirred in MeOH (100 mL) for 24 h. The powder was then filtered and dried under vacuum overnight. The stability of CHD to the entrapment procedure was tested by measuring the spectra of the extract of CHD@Ag in comparison to the spectra of CHD dissolved in MeOH.

II. CHD release profile: Leaching of CHD into HEPES buffer, which was used as the experimental medium for the antibacterial test, was measured by stirring the CHD@Ag composite powder (0.1 g) in HEPES buffer (0.04 M, pH 7.4, 100 mL) and following spectroscopically the release of CHD through its maximum absorption at 255 nm. The scattering of the composite powder was eliminated by subtraction of the absorption at 320 nm from the measured values. Readings were taken every minute for the first 10 min, every 2 min for the successive hour, and then every 5 min for a total of 2.5 h.

Bactericidal Kinetic Tests: The working protocol used was validated and had been practiced in our lab for several years [25] and follows experimental procedures common in the field of biocidal activity testing, in which efficacy kinetics against suspensions of target cells (rather than minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values) are determined. The protocol contains several attributes which substantiate the experimental results. For example, tested bacteria are in the stationary phase which is known to promote higher resistance to stressors, tests are conducted in appropriate mineral buffered systems thus eliminating possible cross reactivity and interferences with the active ingredients, tests are conducted under relevant environmental temperatures (room temperature), and finally, samples are neutralized prior to the enumeration procedure. The latter attribute, which focuses on the prompt deactivation of the active ingredients which assures the annihilation of any residual biocidal activity of the active substances, is crucial in any biocidal testing protocol, especially when efficacy kinetics are determined and even more so when agents that are difficult to neutralize (such as glutaraldehyde) are tested.

A wild-type *Escherichia coli* strain MG1655 (kindly provided by Prof. S. Belkin, the Hebrew University of Jerusalem) was used as a target organism to evaluate the bactericidal efficacy of the active ingredients, i.e., CHD, metallic and ionic Ag, and the CHD@Ag composite. Bacteria were maintained on MacConkey agar plates at 4 °C. Prior to each experiment, an overnight culture was prepared by seeding single colonies into Luria Bertani (LB, 30 mL) broth at 37 °C with shaking. This overnight culture was then washed three times by centrifugation (10 min, 4850 rpm, at 25 °C) and resuspended in HEPES buffer (0.04 M, pH 7.4). The resulting washed pellet was resuspended in HEPES and brought to an optical density (OD_{590}) of 0.3 which corresponds to $\sim 10^8$ colony forming units (CFU) mL^{-1}. Enumeration of bacteria was performed by serial dilution and plating by the pour–plate technique. The inoculated plates were incubated at 37 °C for 24 h and bacterial concentrations were determined by enumerating the resulting CFUs.

The bactericidal kinetic tests were carried out in five acid-washed 500 mL Erlenmeyer flasks filled with HEPES buffer (200 mL). The flasks were capped with dense paper caps to allow oxygen supply. Typically, the active ingredients at the desired concentrations and combinations were added first and then the *E. coli* suspension (2.0 mL) was added to a final concentration of $\sim 10^6$ CFU mL^{-1}. The active ingredients included one of the following: CHD@Ag composite, MeOH-extracted composite, $AgNO_3$ solution, CHD solution, and combinations thereof in final concentrations and amounts summarized in Table 1. The vessels were kept in an incubated

Table 1. Bactericidal compositions concentrations.

Substance name	Concentration [ppm]
Metallic powders	50.0
CHD@Ag composite	50.0
MeOH-extracted composite	50.0
$AgNO_3$ solution (1.2×10^{-6} M)	0.1 [a]
CHD solution (1.5×10^{-6} M)	0.75 [b]

[a] Assuming that metallic silver releases up to 0.2% of Ag^+ [19]. [b] An upper limit of concentration that corresponds to an amount 25% higher than the quantity of CHD that was entrapped within the composite.

shaker at 26 °C under dark conditions. The disinfection kinetics were followed by sequentially sampling aliquots (1.0 mL) from the vessels at the designated times. These samples were neutralized by dilution (1 : 1) with a neutralizing solution ((0.2%w/w sodium thioglycolate, 1.9% w/w sodium thiosulfate, 1% w/w Tween 80, 1.4% w/w lecithin) for 5 min. As previously mentioned, the aim of this neutralization process was to ensure prompt deactivation of any biocidal activity of the tested agents, prior to the enumeration procedure, at the predetermined time intervals. The efficiency of this neutralization procedure was validated in separate control experiments. After neutralization, samples were serially diluted (10 fold) in phosphate buffer saline (PBS) and pour–plated with LB into Petri plates. The plates were incubated at 37 °C for 24 h and bacterial colonies were counted. The bactericidal experiments were repeated at least three times and the mean log reduction values (i.e., $\log(N_t/N_0)$ where N_t = bacterial concentration at time t and N_0 = bacterial concentration at time 0) for the various substances and combinations thereof were plotted versus time.

Instrumentation: TGA was performed with a Mettler–Toledo TGA/SDTA 851e from 50 to 800 °C at a heating rate of 10 °C min^{-1} in flowing dry air. SEM was carried out on a Sirion (FEI) HR-SEM instrument (operating voltage is indicated for each picture). UV-Vis absorption spectroscopy was carried out with a Hewlett–Packard 8452A diode-array UV-Vis spectrophotometer.

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