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Bioinspired Magneto-optical Bacteria

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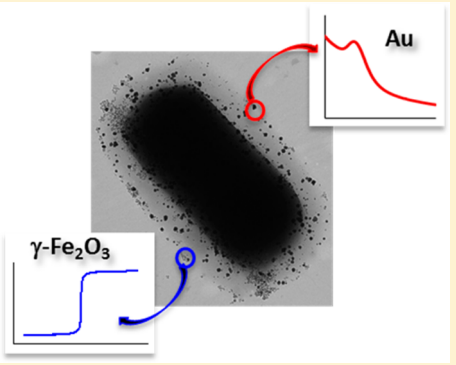
Supporting Information

ABSTRACT: "Two-in-one" magneto-optical bacteria have been produced using the probiotic *Lactobacillus fermentum* for the first time. We took advantage of two features of bacteria to synthesize this novel and bifunctional nanostructure: their metal-reducing properties, to produce gold nanoparticles, and their capacity to incorporate iron oxide nanoparticles at their external surface. The magneto-optical bacteria survive the process and behave as a magnet at room temperature.



INTRODUCTION

Tremendous interest in the possibility of using bifunctional gold-magnetite nanomaterials for biomedical and electronic applications has led to increased research into the synthesis of such materials.[1−8] Synthetic methods usually involve the use of toxic chemicals and high temperatures and pressures, and result in particles that become unstable or aggregate upon interaction with biological media. An alternative approach to traditional synthetic chemistry is the biosynthesis of nanomaterials that employs natural organisms that reduce metal ions into stable nanoparticles.[9−16] Moreover, it should be borne in mind that there is ever-increasing pressure to develop green, eco-friendly, and economically viable synthetic routes to nanomaterials. This has resulted in researchers turning toward biological organisms for inspiration.

Microorganisms, such as bacteria and fungi, can be successfully used for large-scale production of small particles at an extracellular level.[9−16] Biosynthesized nanoparticles usually exhibit enhanced stability and afford better control over morphology. Furthermore, biobased fabrication has been shown to be reproducible and includes the possibility of synthesizing hydrophilic nanoparticles.[17]

Despite these advantages, the use of microorganisms as potential nanoparticle biofactories is a relatively new area of research. Most of the known examples deal with the synthesis of microbial-mediated zerovalent metal nanoparticles, especially gold. Gold nanoparticles are in fact formed by a variety of metal-reducing microorganisms. Although the mechanism has not yet been fully elucidated, it is roughly assumed that the biofilm would capture Au(III) ions on its external surface. The Au(III) ions are then thought to be reduced by biomolecules secreted by the bacteria, producing Au atoms that would aggregate at specific sites to form nanoparticles.[9]

On the other hand, other bacteria are capable of adsorbing, at the extracellular level, amorphous magnetite nanoparticles by a biologically induced process,[18−21] of which the mechanism is still unknown. Similarly, we have recently reported that the direct adhesion of magnetic nanoparticles onto the biofilm of some bacteria is also a viable route for producing novel magnetic bacteria.[22]

Inspired by the existence of both metal-reducing microorganisms capable of producing extracellular gold nanoparticles from the metal cations and microorganisms that can capture iron oxide nanoparticles at an extracellular level, we have designed a new route that incorporates both processes. In this work we describe the preparation of a new type of "'two-in-one"' magneto-optical bacteria based on iron oxide and gold nanoparticles.

The presence of both magnetic and gold nanoparticles in a single nanostructure is a powerful way to combine the properties of two of the most interesting metallic nanobuilding blocks. On one hand, magnetic nanoparticles are of paramount importance in biomedicine as diagnostic tools in magnetic resonance imaging, as mediators for hyperthermic cancer treatment, and as drug-delivery vehicles.[23−26] On the other hand, gold nanoparticles are being employed in biomedicine because of their unique optical, electrical, and photothermal properties.[27−30] In this context, in recent years, a wide range of gold-magnetic nanoparticles have been developed by a variety of physical, chemical, and biological methods, most of them leading to core−shell Au-magnetite nanoparticles.[31−35]

Here we show that bacteria such as *Lactobacillus fermentum*, known to have a positive effect on the maintenance of human

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health since they constitute an important part of natural microbiota, can reduce Au(III) ions to produce discrete extracellular gold nanoparticles and then, in a second step, are able to incorporate iron oxide nanoparticles, also at the external surface, therefore producing bifunctional magneto-optical bacteria. As far as we know, this is the first example of a microorganism simultaneously containing optical gold and magnetic iron oxide nanoparticles.

Furthermore, toxicity assessments showed that neither the gold nor the iron oxide particles were especially toxic or inhibitory to these bacteria. Thus, using this hybrid natural–synthetic approach, we succeeded in obtaining living bacteria that behave as magnets at room temperature and exhibit optical properties.

RESULTS AND DISCUSSION

We found that when an aqueous Au(III) solution was added to a culture of *L. fermentum*, the reaction mixture turned from pale yellow to red within 30 min, indicating the formation of gold nanoparticles (Figure 1). The UV–visible absorption spectrum

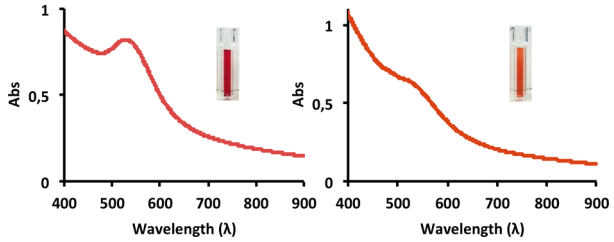

Figure 1. UV–vis spectra of just gold and gold+iron oxide-labeled *Lactobacillus fermentum*.

recorded from the gold-loaded bacteria exhibited a surface plasmon band at 520 nm, which is characteristic of Au nanoparticles, that was not observed for the supernatant after bacteria centrifugation.

To gain further insight into the role of *L. fermentum* in gold nucleation, we chemically reduced Au(III) in the absence of the bacteria, but in the presence of the extracellular reductant solution generated by the cultivated bacteria. For this purpose, *L. fermentum* were cultivated and centrifuged, and the supernatant solution was isolated. Interestingly, when an aqueous Au(III) solution was added to the supernatant extracted from the *L. fermentum* culture, the reaction mixture changed from pale yellow to produce different colors within 1 h and finally formed a black precipitate, indicating the formation of gold aggregates. On the basis of these observations, it can be deduced that gold particles are likely produced with the aid of bacterial extracellular reducing agents. In parallel, they also suggest the existence of a chemical site of gold nucleation on the external bacterial surface. This would explain the stability of the gold nanoparticles once incorporated onto the bacteria and also the absence of any size (or color) evolution with time. Purification and physicochemical characterization of the biomolecules involved in the microbial synthesis of gold nanoparticles need to be investigated. Further analytical and proteomic studies are currently being conducted in order to attain a thorough understanding of the mechanism and nature of the reducing agent(s) and the nucleation site.

Having isolated the gold nanoparticle-loaded bacteria, they can serve as precursors for the incorporation of iron oxide nanoparticles, thus incorporating magnetic properties to thereby obtain the first magneto-optical microorganisms. A liquid culture of gold–*L. fermentum* was incubated with an acidic solution of iron oxide nanoparticles. The resulting bacteria, labeled with gold and iron oxide nanoparticles, were collected by centrifugation, then dispersed in water to form a reddish-brown solution. This solution was examined by UV–vis spectroscopy and transmission electron microscopy (TEM). As shown in Figure 1, the surface plasmon resonance remains practically at the same wavelength, which confirms that gold nanoparticles remain intact after the incorporation of the iron oxide ones. In fact, the supernatant liquid after the final isolation of the magneto-optical bacteria did not contain any gold.

Large accumulations of nanoparticles on the external bacterial surface were revealed by TEM (Figure 2a and b).

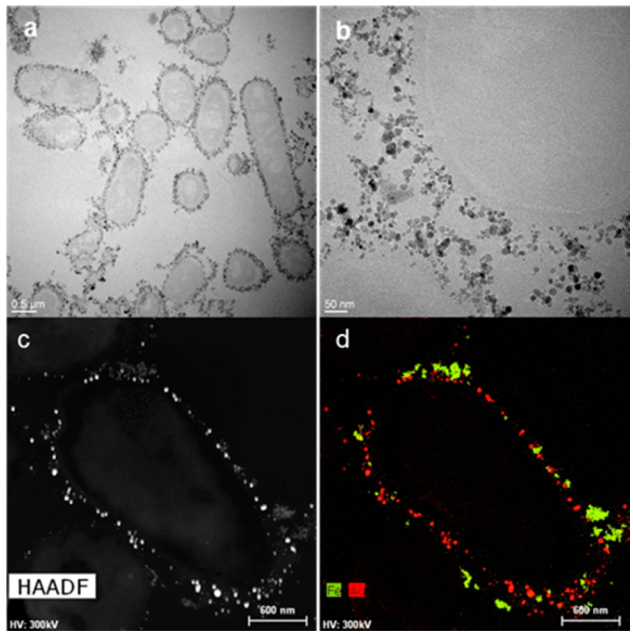

Figure 2. (a) TEM micrograph of a thin epoxy resin section showing the presence of particles at the external surface of the gold+iron oxide-labeled bacteria. (b) An area of (a) at higher magnification, showing the different contrast of gold- and iron-containing nanoparticles. (c) HAADF-STEM micrograph of a single bacterium. (d) EDX compositional maps of iron (green) and gold (red) collected over the whole HAADF-STEM image in (c).

Gold and iron oxide nanoparticles are attached to each bacterium, as demonstrated by high-angle annular dark field scanning transmission electron microscopy (HAADF-STEM) (Figure 2c) and energy-dispersive X-ray spectroscopy (EDX) (Figure 2d). However, iron oxide nanoparticles (in green, Figure 2d) tend to form aggregates, while gold nanoparticles are well dispersed throughout the bacteria surface. The intensity of the HAADF-STEM images depends primarily on the atomic number (Z) and thickness of the specimen. A typical HAADF image of the gold+iron oxide-labeled bacteria (Figure 2c) shows clear evidence of a different contrast between gold- and iron-containing nanoparticles, which serves to distinguish between the two kinds of particles. Every bacterium contains less-bright particles, corresponding to iron oxide structures with a lower Z, and brighter particles, corresponding to the gold nanoblocks. The presence of both gold and iron oxide nanoparticles on each bacterium was unequivocally confirmed

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when inspected by EDX Au and Fe mapping, as can be seen in Figure 2d. Both gold and iron oxide particles had relatively homogeneous size distributions and were approximately spherical. The gold nanoparticles produced were in the size range of 3−18 nm, with an average of 7 ± 2 nm, whereas the iron oxide particles were in the range 6−14 nm, with an average of 10 ± 2 nm.

Figure 3a and b show typical HREM images of agglomerates of iron oxide and gold nanoparticles, respectively, surrounding

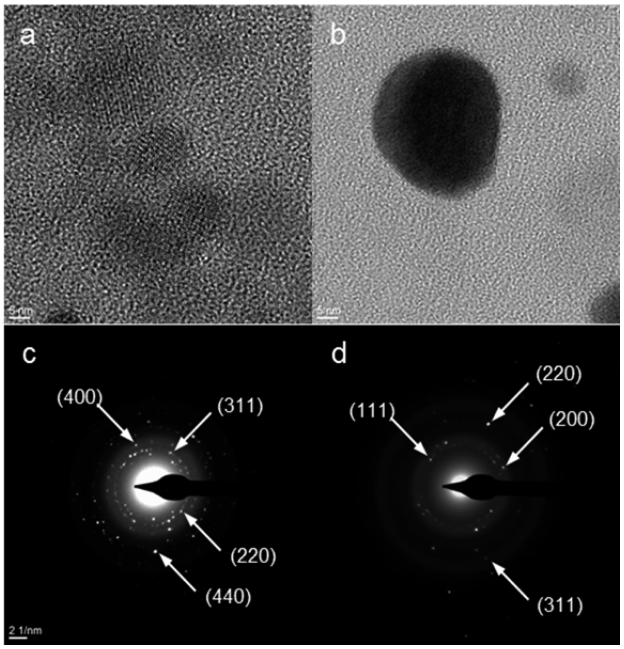

Figure 3. (a) HREM micrograph of iron oxide nanoparticles. (b) HREM micrograph of a single gold nanoparticle. (c) Electron diffraction pattern of iron oxide particles of (a) with labeled reflexions. (d) Electron diffraction pattern of the gold particle of (b) with labeled reflexions.

the bacterial wall. Under high-resolution HREM the lattice fringes of both nanoparticles can be observed, thus confirming their crystalline nature. Measured *d*-spacing and electron diffraction patterns were indexed according to the iron oxide (magnetite/maghemite) and gold structures (Figure 3c and d, respectively).

Figure 4 provides clear evidence that the magneto-optical gold+iron oxide-*L. fermentum* bacteria have ferromagnetic properties at room temperature, as they transfer across a liquid medium placed in the magnetic field of an external magnet. Note that the area of the liquid medium furthest from the magnetic field source is almost colorless. This underlines the fact that both iron oxide and gold nanoparticles are collectively associated in the same bacterial platform. Magnetic studies of lyophilized gold+iron oxide-*L. fermentum* samples were performed using a superconducting quantum interference device (SQUID). Hysteresis loops with coercitivities of 180 Oe at 2 K and 10 Oe at 300 K are observed, indicating ferromagnetism even at room temperature, probably due to magnetic dipolar interactions among iron oxide nanoparticles. The magnetization vs H curves at 2 K and at room temperature showed a sharp increase, reaching saturation at low fields (Figure 4). These results are indicative of a collective ferromagnetic phase both at room temperature and at low

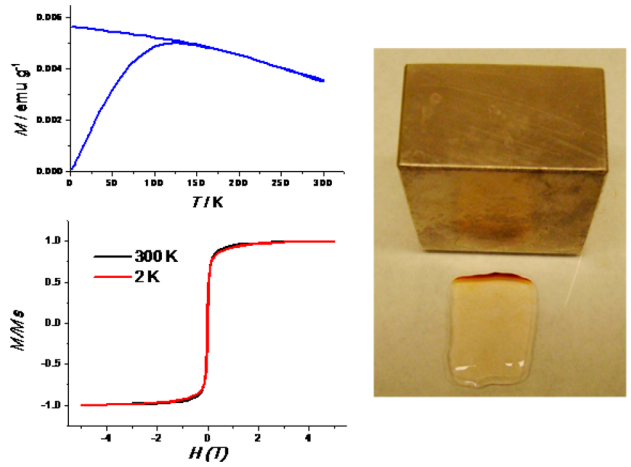

Figure 4. Upper: Field-cooled (FC) and zero-field-cooled (ZFC) curves of lyophilized gold+iron oxide-*Lactobacillus fermentum* powder. Bottom: Hysteresis curves at 300 and 2 K of lyophilized gold+iron oxide-*L. fermentum* powder. Photo: Application of a magnetic field to an aqueous dispersion of gold+maghemite iron oxide-*L. fermentum* produced attraction of the magneto-optical bacteria.

temperatures. This behavior is also apparent from the temperature dependence of the field-cooled (FC) and zero-field-cooled (ZFC) magnetization. The data presented a maximum in the ZFC curve at 130 K, which is generally ascribed to the average blocking temperature of the magnetic moment. Magnetization decreases slightly with increasing temperature but nonetheless shows permanent magnetization at room temperature.

It must be emphasized that isolated iron oxide (magnetite or maghemite) nanoparticles of this size range (10 nm) are superparamagnetic at room temperature and do not show persistent magnetization.[36,37] However, once they are incorporated into the external bacterial surface, dipole−dipole interactions occur due to the close mutual proximity of the iron oxide particles so that the maghemite-gold bacteria behave as ferromagnets at room temperature. This behavior is consistent with results that we have reported previously, in which massive incorporation of iron oxide nanoparticles onto the bacteria surface yielded increased magnetic properties.[22]

Additionally, assessment of the antibacterial activity of these particles revealed that they are nontoxic, nor do they significantly inhibit this kind of bacteria. Quantification of bacterial proliferation was performed using live/dead bacterial viability kits SYTO9 (green) and propidium iodine (red), by counting the number of live (green) and dead (red) bacteria. The average live/dead ratio was used to quantify the effect of gold and iron oxide nanoparticles on bacterial proliferation by comparing with control cultures in the absence of nanoparticles. The presence of nanoparticles resulted in a slight decrease of the live/dead ratio of 15−25% with respect to control experiments.

■ CONCLUSIONS

As conclusions, it must first be emphasized that the putative potential of probiotic bacteria for the biosynthesis of metal nanoparticles is still a relatively unexplored field. Here we describe the first demonstration of the biofabrication of discrete gold nanoparticles using the metal-reducing *L. fermentum* bacterial strain. The resulting gold-loaded bacteria can be used, in a second step, as a precursor for the incorporation of iron

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oxide nanoparticles, thus producing for the first time living bacteria that behave as magnets at room temperature and that also exhibit optical properties.

The biosynthesized magneto-optical nanoparticles built around these kinds of organisms may benefit from their large-scale production and perhaps from their implementation for various biomedical applications through their inclusion in food, where probiotic bacteria are incorporated since they confer health benefits.

EXPERIMENTAL SECTION

Quantification of bacteria proliferation was performed by using the live/dead bacterial viability kits SYTO9 (green) and propidium iodine (red) (Invitrogen), counting the number of live (green) and dead (red) bacteria in a batch of three experiments with the software Image-Pro Plus 6.0. The average live/dead ratio was used to quantify the bacteria proliferation by comparing with control experiments where no nanoparticles were present. The presence of iron oxide and gold nanoparticles resulted in a decrease of the live/dead ratio of 15−25% for three independent experiments carried out in Hank's balanced salt solution (Figure SI).

Liquid cultures of *L. fermentum* were grown in MRS broth (Panreac, 413785) at 37 °C with orbital agitation for 24 h until early stationary phase. Bacteria were then collected via centrifugation and resuspended in Hank's balanced salt solution to a final concentration of 1 mg/mL (3.5 × 108 cfu/mL). A Au(III) aqueous solution (gold tetrachloride, Sigma-Aldrich, 99%) was dissolved in Type 1 Milli-Q ultrapure water and then added to the bacterial suspension to give a final gold concentration of 1 mM. The reaction mixture was kept at 37 °C and under continuous stirring for 30 min until the appearance of a characteristic red-wine-colored solution, indicating the formation of gold nanoparticles. The resulting gold−biomass was centrifuged at 100 rpm for 30 min. The supernatant exhibited no band in the visible region. The quantity of gold measured by inductively coupled plasma mass spectrometry was zero. Control reactions in the absence of *L. fermentum*, where the culture supernatants of Hank's balanced salt solution and MRS broth both exhibited no color change or absorbance at 520 nm, clearly indicated that the presence of bacteria in the biosynthesis of gold nanoparticles is a prerequisite. An acidic solution (pH 2) of maghemite nanoparticles (1 μL, at 0.95−1 M of iron), prepared as previously reported,[36,37] was added to the gold-loaded *L. fermentum* and maintained at 37 °C under orbital agitation for 30 min. The reaction mixture was washed with several centrifugation cycles (100 rpm, 5 °C, 30 min) to remove any unbound iron oxide nanoparticles, and the final bacterial pellet resuspended in a 50 mM sodium citrate, PBS, Milli-Q water 1:1:4 mixture at pH 5. The resulting gold+iron oxide-*L. fermentum* suspension remained stable at room temperature.

The sample of gold+iron oxide-*L. fermentum* was embedded in an epoxy resin. Fixation was achieved by adding 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer solution at 4 °C for 4 h. The sample was washed three times in 0.1 M sodium cacodylate buffer for 15 min. Then, drying cycles with ethanol and propylene oxide were applied. Finally, the sample was embedded in an epoxy resin and left overnight at 4 °C. After ultramicrocutting, samples were observed with a FEI TITAN G2 microscope.

ASSOCIATED CONTENT

Supporting Information

Fluorescence microscopy images of magneto-optical *Lactobacillus fermentum* and proliferation obtained using a live/dead bacterial viability kit. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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