Biologically Functional Cationic Phospholipid–Gold Nanoplasmonic Carriers

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Abstract: Biologically functional cationic phospholipid–gold nanoplasmonic carriers have been designed to simultaneously exhibit carrier capabilities, demonstrate improved colloidal stability, and show no cytotoxicity under physiological conditions. These carriers are able to retain their unique nanoscale optical properties under physiological conditions, making them particularly useful in a wide range of imaging, therapeutic, and gene delivery applications that utilize selective nanoplasmonic properties.

Introduction

Gold nanoparticles (GNPs) in the near-infrared (NIR) spectral region, due to their size and core material, display unique optical properties that make them attractive candidates for drug delivery,1–4 gene delivery,5–7 biomedical and molecular imaging,8–11 and therapeutic.12–20 Because of their large surface area, GNPs are ideal carriers of biomolecules for these applications. While attached to carriers, biomolecules are in an inactive state. When GNP carriers are specifically used to convert light into heat, otherwise known as photothermal conversion,21–23 released biomolecules enter an active state to freely interact with the environment. Such optically activated GNP carriers are referred to as nanoplasmonic carriers.

In particular, the NIR wavelength regime is well suited for biomedical applications because tissues and cells are essentially transparent at 800–1300 nm.24,25 It is possible to obtain very efficient photothermal conversion of energy when the NIR light is matched to the plasmon resonance wavelength of the GNP. Additionally, heat transfer from the surface of GNPs to the surrounding cellular environment is highly localized, decaying exponentially within a few nanometers,7,21,26,27 and therefore is thought to have minimal adverse effects on cells. Among the GNPs, rod-shaped GNPs, known as nanorods, are of particular interest due to their large absorption cross-section, a narrow spectral width of the longitudinal plasmon resonance band, and tunability of the longitudinal plasmon resonance wavelength based on aspect ratio. The unique optical properties of gold nanorods arise due to their nanoscale asymmetric geometry and gold core material.

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Despite these unique optical properties, the combination of three key factors, carrier functionality, colloidal stability, and cytotoxicity, has hindered the widespread use of gold nanorods as carriers in biological and biomedical applications. Therefore, to harness the full potential of nanomaterials in these applications, the surface coating material as well as the gold core material deserves attention. With respect to cytotoxicity, while the gold core is widely accepted as being biocompatible, bare gold nanoparticles are known to interact with proteins and induce misfolding at physiological conditions. Specifically, in the case of gold nanorods, the asymmetric geometry is obtained by synthesizing the nanorods in the presence of a high concentration (>0.1 M) of cetyltrimethylammonium bromide (CTAB), a cationic micellar surfactant that associates preferentially with the {110} crystallographic facet of gold. However, CTAB is known to degrade biomembranes and peptides, raising significant concern about the cytotoxicity of CTAB-coated nanorods in vitro and in vivo.

The cytotoxic effects of CTAB-coated nanorods can be minimized by reducing the CTAB concentration below the critical micellar concentration, but at the expense of the nanorod suspension stability (Figure 1b), consequently compromising their unique optical properties in biological environments. Notably, under physiological conditions, aggregation of nanoparticles has been shown to significantly red-shift and decrease the amplitude of the plasmon resonance band due to closely interacting nanoparticles. Finally, for plasmon-resonant nanorods to function as biological carriers, biomolecules such as DNA oligonucleotides, RNA oligonucleotides, or small interfering RNA (siRNA) must be able to adsorb on their surface. Notably, in the case of siRNA, chemical modifications may affect the functionality and efficacy of siRNA. Therefore, the attachment of siRNA onto the surface of gold nanorods without additional chemical modifications to the siRNA themselves is highly desirable. Poly(ethylene glycol) (PEG) coating is widely utilized for this purpose, to improve colloidal stability and reduce nonspecific adsorption.
glycol (PEG) has previously been used to modify gold nanorods for in vivo applications; however, their ability to maximally carry subsequent biomolecules is limited due to the absence of surface charge. Organothiols and poly(styrene)sulfonate have been used to coat gold nanorods; however, attachment of nucleic acids has not been demonstrated. Synthetic lipids have also been used to modify gold nanorods; however, carrier capabilities and/or biocompatibility have not yet been addressed. Polyelectrolyte coating schemes have also been used to coat gold nanorods; however, plasmmonic properties under physiological conditions have not yet been discussed. Alternatively, because cationic lipid formulations have already been optimized for both in vitro and in vivo gene transfer over the past decade and many cationic lipid-based gene delivery approaches are currently being tested at the clinical level, validated cationic lipids make ideal candidates for modifying ordinary nanorods into biocompatible nanorods that simultaneously satisfy carrier functionality, colloidal stability, and nontoxicity. The positively charged surface can be used to adsorb negatively charged biomolecules such as RNA oligonucleotides, siRNA, or DNA oligonucleotides (Figure 1b). In addition to carrying siRNA, RNA oligonucleotides, and DNA oligonucleotides, these carriers can also be used to carry a variety of other compounds such as proteins and drugs by incorporation into or binding to the cationic phospholipid membrane (Figure 1a).

In this Article, we present biologically functional cationic phospholipid-gold plasmonic carriers (bioGNPs) that simultaneously exhibit carrier capabilities, demonstrate improved colloidal stability, maintain plasmonic properties, and show no cytotoxicity under physiological conditions. Using an adaptation of vesicle-to-nanoparticle fusion, we exchanged the cytoxicity CTAB surfactant at the nanorod surface with commercially available cationic phospholipids (Figure 1b) successfully used for in vivo studies. We first demonstrate that bioGNPs are stable under physiological conditions, thus retaining their unique plasmonic properties. We then show that the positively charged surface of nanorods can adsorb cargo such as DNA oligonucleotides, RNA oligonucleotides, or siRNA. We finally demonstrate the biocompatibility of bioGNPs via viability/cytotoxicity and cell proliferation studies.

Experimental Procedures

Cell Preparation. The human breast carcinoma line MCF-7 was purchased from the American type Culture Collection (ATCC). Dulbecco’s modified eagle’s media (DMEM) was purchased from Invitrogen and was supplemented with 10% heat-inactivated fetal bovine serum, 0.1% nonessential amino acids, and 1% sodium pyruvate. Cells were cultured in the supplemented media and maintained in a 37 °C incubator with 5% CO2 humidified air.

Synthesis of D Nano/RNA-Facilitated Rod-Shaped GNP s. Gold nanorods of aspect ratio 3.0 were synthesized by adapting a previously reported seed-mediated growth method to a DNase/RNAse-free environment. Hexadecyltrimethylammonium bromide (CTAB), silver nitrate (AgNO3), l-ascorbic acid, sodium tetrahydrolorate (NaBH4), and hydrogentetrachloroaurate (HAuCl4) were purchased from Alfa Aesar. All solutions were prepared using 0.2 µm filtered nuclease-free water. All glassware and metalware were baked at 240 °C for 24 h to remove exogenous RNAse. All pipetting devices and counter space was treated with 70% ethanol. All disposable pipet tips and centrifuge tubes were certified to be free of RNAse.

To prepare the seed solution, 5 mL of 0.2 M CTAB solution was mixed with 5 mL of 0.0005 M HAuCl4. Ice-cold 0.010 M NaBH4 (0.60 mL) was then added, and the solution was continuously stirred for 2 min at room temperature. To prepare the growth solution, 9.5 mL of 0.1 M CTAB was mixed with 60 µL of 0.10 M AgNO3, 0.5 mL of 0.01 M HAuCl4, 55 µL of 0.10 M ascorbic acid, and 12 µL of seed solution with continuous stirring. The gold nanorods were aged overnight at room temperature. At this point, the CTAB concentration in the gold nanorod solution was approximately 0.1 M. The nanorod concentration (approximately 30 µg/mL or 7E11 particles/mL) was confirmed by adjusting to an absorbance of 1 at the longitudinal plasmon resonance wavelength using UV–vis spectroscopy. Aspect ratio was determined by scanning electron microscopy (Hitachi S-4500 FESEM) at 150 000× magnification.

Synthesis of BioGNPs. Commercially available cationic phospholipids Oligofectamine, Lipofectamine 2000, and sc29528 were purchased from Invitrogen and Santa Cruz Biotechnology. Nonionic surfactant Brij56 was purchased from Fluka and prepared in nuclease-free water.

To remove excess CTAB surfactant, 500 µL of unmodified CTAB-coated nanorods (UV–vis absorbance of 1) in 0.1 M CTAB was centrifuged at 5000 rpm for 10 min. A 10 µL pellet was transferred to a new microcentrifuge tube, redispersed in 500 µL of nuclease-free water such that the final concentration was 0.1 mM CTAB FAM-3′, guaGUCUU-FAM-3′ were purchased from Integrated DNA Technologies. The sequence was: S’GUAGAUAAUACCAG-GAGGCUCU-FAM-3′. Rnaase-free 20X Tris-EDTA (TE) buffer was prepared.

purchased from Invitrogen and was used to prepare 1X TE buffer solution using nuclease-free water.

Stock solutions of RNA oligonucleotides were prepared in RNase-free 1X TE buffer. To 500 µL of bioGNPs was added 0.25 µL of 100 µM RNA oligonucleotides. The solution was vortexed and allowed to incubate for 1 h. To remove excess oligonucleotides from solution, bioGNPs were washed twice with nuclease-free water by centrifugation at 5000 rpm for 10 min. After preparation of RNA–bioGNP conjugates, an absorbance of 0.2 was measured by UV–vis. By comparing with the original nanorod’s UV–vis absorbance of 1, the concentration of bioGNPs was estimated to be approximately 1/5 the original nanorod concentration (approximately 6 µg/mL or 1.4E11 particles/mL). Because fluorescence quenching by bioGNPs was not observed, bioGNP carriers of fluorescently labeled RNA oligonucleotides were finally visualized using fluorescence microscopy.

Dynamic Light Scattering of BioGNP Carriers. DLS data were collected for all samples using a DynaPro-99-E 15 LS apparatus from Wyatt Technologies. For each sample, at least 20 scans were performed, each with a 10 s acquisition time, using a scattering angle of 90°. Optimum medium was purchased from Invitrogen.

CTAB-coated nanorods, Brij56-coated nanorods, and bioGNPs were prepared as described above. DLS measurements were taken of each sample (diluted 1:60 in a quartz cuvette) as-prepared, after 0.5% bovine serum albumin (BSA) and 0.1% sodium azide in 1X BD Biosciences. Cells were harvested using 1 mM EDTA for 10 min. 1X PBS was then added to each well to wash the cells. A 5 µL pellet by centrifugation at 5000 rpm for 10 min. MCF-7 cells were harvested and resuspended in DMEM media to a concentration of 240 000 cells/mL. To each well were added 0.5 mL of cell suspension and 5 µL of concentrated bioGNPs or unmodified CTAB-coated nanorods (120 000 cells/well). The cells were allowed to incubate for 24 h. After 24 h, wells containing cells that were to be analyzed were exchanged with fresh media. Cells that were to be analyzed after 24 h were washed with 1X PBS, detached using 0.25% trypsin for 1 min, and resuspended in 2% paraformaldehyde. Trypan blue was then finally added to the cell suspension at 50% concentration to quench uninternalized bioGNP carriers.

As a control, surface receptor ERBB2 on ERBB2-positive BT474 cells was labeled with antibodies conjugated to FITC dye (340553, BD Biosciences). Cells were harvested using 1 mM EDTA for 10 min. Cells were resuspended in 1X PBS containing 2% paraformaldehyde and 0.5% bovine serum albumin (BSA) and 0.1% sodium azide in 1X PBS. Five microliters of normal mouse IgG (I8765, Sigma Aldrich) was added, and cells were incubated for 15 min to prevent nonspecific binding of antibodies to Fc receptors. Fifteen microliters of anti-ERBB2-FITC was added, and cells were incubated for 45 min at room temperature. Cells were then washed in surface-staining buffer. Cells were resuspended in 1X PBS containing 2% paraformaldehyde. Trypan blue was then finally added to the cell suspension at 50% concentration to quench surface receptors labeled with fluorescent antibodies. LSRII flow cytometer (BD Biosciences) and FlowJo software (Tree Star, Ashland, Oregon) were used to analyze samples.

Visualization of Internalized BioGNP Carriers by Dark-field Light Microscopy. 500 µL of bioGNPs was concentrated into a 5 µL pellet by centrifugation at 5000 rpm for 10 min. MCF-7 cells were harvested and resuspended in DMEM media to a concentration of 240 000 cells/mL. To each well were added 0.5 mL of cell suspension and 5 µL of concentrated bioGNPs or unmodified CTAB-coated nanorods (120 000 cells/well). The cells were allowed to incubate for 24 h. After 24 h, wells containing cells that were to be analyzed were exchanged with fresh media. Cells that were to be analyzed after 24 h were washed with 1X PBS, detached using 0.25% trypsin for 1 min, and resuspended in 2% paraformaldehyde. Trypan blue was then finally added to the cell suspension at 50% concentration to quench uninternalized bioGNP carriers.

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Results and Discussion

Gold nanorod carriers were synthesized by modifying a seed-mediated growth approach29,55 to be free of DNase/RNase contamination. Using dynamic light scattering (DLS), in Figure 2a and Supporting Information Figure S2, the resultant CTAB-coated nanorod carriers show a size distribution with an average radius of ~15 nm (30 nm diameter). Because the nanorod’s length was short and the rotational diffusion was therefore rapid, the nanorod was approximated as a translationally diffusing sphere whose diameter equaled the length of the nanorod (30 nm). Lengths based on DLS measurements were in agreement with lengths seen in SEM images (Figure 4a). Because the resultant gold nanorod carriers were coated with CTAB that yielded a net positive surface charge from the quaternary ammonium surfactant headgroup,29 negatively charged, fluorescently conjugated 21-mer RNA oligonucleotides readily attached to the CTAB-coated nanorod carriers, enabling visualization by fluorescent microscopy (Figure 2b). As shown in Supporting Information Figure S4, while CTAB-coated nanorods were relatively stable and showed a narrow distribution before and after RNA incorporation, an additional population with an average radius of 115 nm appeared in the size distribution after RNA incorporation, suggesting the possible onset of aggregation of the RNA-CTAB-coated nanorods.

The first stage in demonstrating stable bioGNPs was place-exchanging CTAB with cationic phospholipids vesicles at the nanorod surface by using a vesicle-to-nanorod fusion approach.31 Excess CTAB surfactant was first removed from the CTAB-coated nanorod solution to yield a final CTAB concentration of 0.1 mM. These CTAB-coated nanorods were...
then dispersed in various commercially available liposome formulations in approximately <50 fold excess. The phospholipid bilayer coating at the nanorods’ surface readily replaced the CTAB surfactant (Figure 1b). The formation of phospholipid bilayer coatings on gold nanorods and simultaneous loss of surfactant coatings have been previously determined by NMR and FTIR spectroscopy and zeta potential measurements. An alternative route to phospholipid-coated nanorods was to first place-exchange the CTAB surfactant with the nonionic surfactant Brij56, followed by place-exchange with cationic phospholipid vesicles. Because CTAB removal from the nanorods is essential to minimize cytotoxicity and to eliminate any electrostatic contribution, the latter procedure was developed in order to develop phospholipid coatings on gold nanorods and simultaneously loss of surfactant coatings. The smaller average lengths suggest that the nonionic surfactant Brij56 is essential to minimize cytotoxicity and to eliminate any electrostatic contribution, the latter procedure was developed in order to develop phospholipid coatings on gold nanorods and simultaneously loss of surfactant coatings.46

Figure 2. Synthesis of bioGNP carriers. (a) CTAB-coated nanorod carriers are prepared using seed-mediated growth process. Using dynamic light scattering (DLS), CTAB-coated nanorod carriers show a size distribution with an average radius of ~15 nm (30 nm diameter). (b) Fluorescent image showing CTAB-coated nanorods carry negatively charged, fluorescent FAM-conjugated 21-mer RNA oligonucleotides. (c) The CTAB surfactant is then place-exchanged with a nonionic surfactant Brij56 surfactant. Using DLS, Brij56-coated nanorods show a size distribution with an average radius ~50 nm (100 nm length). (d) Fluorescent image showing that Brij56-coated nanorods are resistant to coupling with fluorescently conjugated RNA oligonucleotides. The Brij56 coating was then place-exchanged with cationic phospholipids to form bioGNPs. Using DLS, bioGNPs show an average radius of ~15 nm (30 nm length) with a narrow size distribution. (f) Fluorescent image showing bioGNPs carry negatively charged, fluorescent FAM-conjugated RNA oligonucleotides.


size distribution (Figure 2e and Supporting Information Figure S1 and S2). The smaller average lengths suggest that the phospholipid bilayer did not layer on top of the Brij56 coating, but instead successfully place-exchanged with the Brij56 at the bioGNPs’ surface. Furthermore, negatively charged, fluorescently conjugated RNA oligonucleotides readily attach to the bioGNP carriers, strongly suggesting that the nonionic surfactant was successfully place-exchanged by a positively charged phospholipid bilayer (Figure 2f and Supporting Information Figure S3). As shown in Supporting Information Figure S4, bioGNPs showed a narrow distribution before and after RNA incorporation, demonstrating that bioGNP carriers were stable after RNA incorporation. To estimate the amount of RNA attached to bioGNP carriers, fluorescently conjugated RNA oligonucleotides were first attached to bioGNP carriers (approximately 1E11 particles/mL based on UV–vis measurements), and unbound oligonucleotides were subsequently removed from the background solution by centrifugation. Using Triton X-100 detergent to disrupt the cationic phospholipid bilayer around the bioGNP carriers, the bound oligonucleotides were then released from the bioGNP carriers into solution and the fluorescent intensity was measured. To calibrate the fluorescent intensity to the concentration of oligonucleotides, the fluorescent intensities of known concentrations of fluorescently conjugated oligonucleotides were measured (Supporting Information Figure S5). As seen in Supporting Information Figure S5, bioGNPs (approximately 1E11 particles/mL) released approximately 0.03 µM RNA oligonucleotides after treatment with Triton X-100 detergent.
Having established that the CTAB coating can be exchanged with a cationic phospholipid membrane, the stability of bioGNPs was then studied and compared against the stability of the CTAB-coated nanorods under biological conditions. CTAB-coated nanorods, which were washed twice and resuspended in nuclease-free water, showed an average radius of ∼15 nm (30 nm length) with a broad distribution in Figure 3c. The twice-washed CTAB-coated nanorods showed a slightly wider size distribution as compared to as-prepared CTAB-coated nanorods in 0.1 M CTAB solution (Figure 3b), suggesting the onset of aggregation. After resuspending the twice-washed CTAB-coated particles in cell culture media, further broadening was seen of the size distribution centered at radius of 60 nm, suggesting that particles were highly unstable in culture media (Figure 3d). In comparison, bioGNPs that were washed twice and resuspended in nuclease-free water were highly stable (Figure 2g). When resuspended in cell culture media, bioGNPs continue to exhibit a narrow size distribution (Figure 2h and Figure S1), thus affirming their excellent stability under biological conditions.

Having confirmed bioGNPs’ stability, we then investigated the internalization of bioGNPs into human cells. Net-positively charged bioGNPs were endocytosed by MCF-7 human breast carcinoma cells by incubation for 10 h. To visualize internalized bioGNPs, the cells were then illuminated with unpolarized white light from an oblique angle using a darkfield condenser lens, and scattered light was collected using a transmission-mode darkfield microscope (Figure 4b). To locate cells’ nuclei, darkfield scattering images were overlaid with DAPI-stained images. It is clearly evident from Figure 4b that scattered light from cells containing bioGNPs is easily differentiated from that from cells lacking bioGNPs.

To further confirm that bioGNPs were in fact internalized within cells and not externally adsorbed onto the cells’ surface, MCF-7 cells were first exposed for 10 h to bioGNP carriers of fluorescently labeled RNA oligonucleotides. Uninternalized carriers were then rendered nonfluorescent by using trypan blue as a quencher. Because trypan blue absorbs light between 475 and 675 nm, the emission of fluorescent dyes within this wavelength range is quenched in the presence of trypan blue. If bioGNP carriers of FAM-labeled RNA oligonucleotides (excitation 495 nm, emission 520 nm) are indeed internalized within cells, it is expected that their fluorescence should remain unquenched when cells are resuspended in trypan blue. Flow

Unmodified CTAB-coated nanorods were therefore initially phospholipids was estimated to be approximately 6 mM based cell cytotoxicity. The concentration of commercial cationic membranes of compromised cells, was used as a measure of Propidium iodide (PI), a dye that enters through permeabilized enzymes in living cells, was used as a measure of cell viability. fluorescent cell-impermeant calcein by intracellular esterase converts from nonfluorescent cell-permeant calcein AM into mine numbers of live and dead cells. Calcein AM, a dye that using a two-color fluorescence assay to simultaneously deter- conducting a long-term viability/cytotoxicity and proliferation studies in MCF-7 cells. Viability/cytotoxicity was conducted in the presence of trypan blue (Figure 4d).

Finally, the biocompatibility of bioGNPs was investigated and compared against unmodified CTAB-coated nanorods by conducting a long-term viability/cytotoxicity and proliferation studies in MCF-7 cells. Viability/cytotoxicity was conducted using a two-color fluorescence assay to simultaneously determine numbers of live and dead cells. Cellaein AM, a dye that converts from nonfluorescent cell-permeant calcein AM into fluorescent cell-impermeant calcein by intracellular esterase enzymes in living cells, was used as a measure of cell viability. Propidium iodide (PI), a dye that enters through permeabilized membranes of compromised cells, was used as a measure of cell cytotoxicity. The concentration of commercial cationic phospholipids was estimated to be approximately 6 mM based on DLS comparison with standards of known concentration. Unmodified CTAB-coated nanorods were therefore initially resuspended in 6 mM CTAB to obtain comparable results. Unmodified CTAB-coated nanorods and bioGNPs were then washed with nuclease-free water to remove excess lipids/CTAB and resuspended in cell culture media. Based on DLS measurements, bioGNPs were highly stable while unmodified CTAB-coated nanorods were only moderately stable in cell culture media. To retain unique optical properties of stable nanoparticles, further washing was avoided because twice-washed CTAB-coated nanorods tend to strongly aggregate in cell culture media as seen in DLS measurements in Figure 3d. After 72 and 120 h of incubation, based on flow cytometric data, viability (Figure 5a) and cytotoxicity (Figure 5b) of cells containing bioGNPs were statistically significant from cells containing unmodified CTAB-coated nanorods (also see Supporting Information Figure S6). It is apparent that cells containing bioGNPs are viable, whereas cells containing CTAB-coated nanorods are compromised. Clearly, bioGNP carriers are a superior alternative to unmodified CTAB-coated nanorods for biological studies.

Cell proliferation was also assessed by cell count analysis after 120 h of incubation with and without bioGNPs. Based on cell count analysis, doubling times were then determined for bioGNP-containing samples and compared to an untreated control sample. Doubling time of 55 h for the untreated control sample was in agreement with doubling times previously determined for CTAB-coated nanorods for biological studies.
reported in literature for MCF-7 cells. Cells containing bioGNPs exhibited doubling times approximately similar to that of the untreated control sample (Figure 5c), verifying that incubation with bioGNPs did not adversely affect proliferation of MCF-7 cells.

While in vitro experiments were successfully demonstrated here, we anticipate developing bioGNP carriers for use in vivo mammalian and nonmammalian model systems. BioGNP carriers can be coated with cationic phospholipid formulations specifically optimized for biodistribution in vivo. In addition to carrying siRNA, RNA oligonucleotides, and DNA oligonucleotides for gene delivery applications, bioGNPs can also be used to carry a variety of other compounds such as proteins and drugs for in vivo applications.

Conclusions

In closing, we have designed biologically functional phospholipid–gold plasmonic bioGNP carriers that exhibit carrier capabilities, demonstrate improved nanoparticle stability, and show no cytotoxicity under physiological conditions. Successful demonstration of these advantages has been shown here using mammalian cell lines. In addition to these advantages, because bioGNPs are able to retain their unique optical properties under physiological conditions, we anticipate that bioGNPs will be particularly useful in a wide range of applications that utilize selective nanoplasmonic properties. We therefore expect bioGNPs to have important implications in advancing drug delivery, gene delivery, biomedical and molecular imaging, and therapeutics.

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Supporting Information Available: Supporting DLS measurements, additional fluorescent images after FAM-RNA incorporation onto bioGNPs, experimental estimation of amount of RNA on bioGNPs, and additional flow cytometric data. Complete ref 50. This material is available free of charge via the Internet at http://pubs.acs.org.