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Gold Nanocages Entering into the Realm of High-contrast Photoacoustic Ocular Imaging

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ABSTRACT

Bioinert gold nanoparticles of various shapes and functionalities are widely accepted as contrast agents (CAs) for several modalities of imaging, viz., electron microscopy, computerized tomography (CT), X-ray and photoacoustic (PA) imaging. However, testing of novel compact-imaging probes for ocular diagnostic imaging is always challenging. Here, ultra-fast microwave oven synthesized gold nanocages (AuNcgs) were successfully demonstrated for high-contrast PA ocular imaging for the first time. Methods are described for the synthesis, characterization and application of quickly- synthesized AuNcgs in diagnostic ocular imaging. PA and ultrasound (US) images were acquired using a commercial US imaging scanner integrated with a tunable nanosecond pulsed laser. This integrated hybrid-modality system is a combined PA and US platform for imaging that enabled acquiring of complementary structural and optical absorption-based information simultaneously. Initial experiments were conducted using tubings filled with solutions of different concentrations of quickly- synthesized AuNcgs. Biological PA and US imaging were demonstrated using enucleated porcine eye samples. Based on the acquired results it is envisaged that AuNcgs can be employed as a high strength PA CA to potentially diagnose ocular disease like uveal melanoma in the near future.

Key Words: gold nanocages; photoacoustic imaging; ocular imaging; nanoparticles; high-contrast agents; bioimaging

1. Introduction

Ocular imaging is highly critical for the diagnosis, therapy and analysis of the severity and progression of eye diseases¹. Several modalities in optical imaging are supplementing the most established ophthalmic imaging techniques like ultrasonography^{2,3}, angiography², and fundus photography.¹ Moreover, development of ophthalmic photoacoustic imaging (PAI) has demonstrated a safe and high-resolution visualization of deep tissue structures of eye including the retina, choroid and optic nerve.² PAI is a fast-growing biomedical imaging technique that has been configured to overcome the penetration depth limit of optical imaging modalities, which was of the order of ~1 mm in human tissues or skin⁴. Recently, PAI has been reported to perform deep tissue imaging of up to several centimeters^{5,6}.

PAI has been employed in biomedical imaging for various applications as it provides rich contrast based on the absorption heterogeneities⁷. The complementary structural and functional information provided by PAI can help clinicians to make accurate disease diagnosis. Blood is a common photoacoustic (PA) endogenous absorbers⁸, which can be imaged to reveal tissue vasculature⁹ and determine total hemoglobin concentration and saturation of blood oxygen.^{8,10} Several exogenous contrast agents (CAs) like nanoparticles,^{11–13} organic dyes¹⁴ and fluorescent proteins¹⁵ are also used to provide enhanced contrast of specific targets during PAI. Gold (Au) is commonly used as the coating material for biomedical PAI as it is biocompatible.^{12,16} The peak absorption of such metal nanoparticles can be tuned over a broad spectrum from the near-ultraviolet to the mid-infrared,^{12,17} by several ways such as altering its shape¹⁴ (aspect ratio) and the relative dimensions of the core and shell.^{17,18}

PA phenomenon is a hybrid combination of optical excitation and acoustic detection. It occurs when the sample absorbs some of the energy from the pulsed ultraviolet (UV), visible light or near-infrared excitation.¹⁹ The energy absorbed by the sample causes an increase in transient temperature, followed by thermoelastic expansion. This produces an initial pressure rise forming broadband acoustic waves (PA waves),^{8,18,20} which can be detected by an ultrasound transducer (UST). The initial pressure rise P_0 can be described using:

$$P_0(\lambda) = \Gamma F(\lambda) \mu_a(\lambda), \quad (1)$$

where P_0 is directly proportional to the unitless Grüneisen parameter Γ , fluence F of the excitation wavelength λ , and optical absorption coefficient μ_a .^{18,21}

Here, we are using gold nanocages (AuNcgs) synthesized via ultra-fast microwave assisted method²² as PA CA in ocular imaging for the first time, demonstrated using enucleated porcine eye

models. Conventionally produced AuNcgs have been established as a high-contrast PA agent for imaging tumors and cancers *in vivo*.^{23–27} However, its applications and possibilities in ocular imaging is far overlooked. The time-consuming conventional synthesis of AuNcgs has limited its wide application in optical imaging and theranostic studies. We have developed an ultra-fast synthesis method for AuNcgs using microwave oven heating technology, which drastically reduced the synthesis time from several hours to few seconds.^{22,28} Washed AuNcgs were primarily subjected to several morphological and chemical characterizations prior to acquiring PA images.

In this paper, we have employed the quickly-synthesized AuNcgs developed by our novel method, which are first tested to show that they can generate PA waves under pulsed optical excitation. In order to demonstrate the effect of concentration on PA signal strength, different concentrations of AuNcg solutions were added into separate tubings. Also, the AuNcgs are used as PA CA in ocular imaging for the first time and demonstrated using enucleated porcine eye models. The synthesized AuNcgs are injected into the eye models to simulate and show its potential use as high strength PA CAs for disease diagnosis of diseases, such as uveal melanoma. Uveal melanoma is a type of intraocular cancer that is generally found in the iris, choroid, and ciliary body. An early diagnosis of this condition can help the patient from developing painful eye condition leading to vision loss and in certain cases of deaths due to metastasis.^{29,30} It was reported that bioconjugated AuNcgs were demonstrated for its use as CAs in cancer cell imaging.³⁰

2. Results and Discussion

Synthesis and Characterization of Quickly-synthesized AuNcgs

On reacting AgNcbs with 5 ml of 0.1 mM HAuCl₄ solution, pale-violet colored AuNcg solution was obtained. Highly monodispersed hollow AuNcgs were harvested after microwave assisted synthesis and several washing steps. Figure 1 illustrates the morphological and structural characterization of AuNcgs using high-resolution transmission electron microscopy (HRTEM). Figure 1a shows the HRTEM image of an individual AuNcg with holes in the faces and corners with corresponding fast Fourier transform (FFT) image being shown in the inset. Transmission electron microscopy (TEM) characterization revealed the hollow nature of the AuNcg showing a thick-walled cubical box pattern. The wall thickness of AuNcgs was measured to be 5 ± 2 nm with an average edge length of ≈ 65 nm. Atomic resolution image of one of the corners of AuNcg was recorded and presented in Figure 1b. Line profile of the FFT data generated shows the interplane distance of the Au crystal (Fig. 1c). Line profile (Fig. 1d) of the well-defined lattice fringes from the selected area of Figure 1B shows a d-spacing of 2Å, which can be indexed to {200} planes of the Au fcc structure.³¹ Depending upon the degree of passivation by PVP over AuNcgs, the dispersity of the nanocages

121 differ (Fig. 1e). However, on thorough washing and ultrasonication, relatively monodispersed
 122 AuNcgs were collected. Furthermore, high-angle annular dark-field scanning transmission electron
 123 microscopy (HAADF-STEM) images (Figs. 1f-g) allow the evolution of AuNcgs with holes from
 124 solid AgNcbs in a uniform fashion to be monitored. Transmission electron microscopy-energy
 125 dispersive spectroscopy (TEM-EDS) mappings of AuNcgs reveal the homogenous alloy
 126 distribution of Au and Ag (Figs. 1h-k). However, the semi-quantitative assessment of atomic
 127 percentage of Au and Ag shows a successful galvanic displacement of Ag from the initial AgNcb
 128 template. Figure 1m shows the EDS spectrum of constitutive elements present in the AuNcgs
 129 analyzed.

Figure 1: HRTEM characterization of quickly-synthesized AuNcgs. **a**, HRTEM image of AuNcg; Corresponding FFT image for the AuNcg (Inset); **b**, HRTEM image of the corner of AuNcg shown in figure **a**; **c**, Line profile of FFT image shown in figure **a**-inset; **d**, Line profile from the highlighted area of the HRTEM image of AuNcg shown in figure **b**; **e**, low magnification TEM image of AuNcgs in cluster; **f**, High magnification bright field TEM image of AuNcg; **g**, HAADF-STEM image of AuNcg shown in **f**; **h-k**, EDS mapping images for AuNcg: **h**- Bright field; **i**- Au; **j**- Ag; **k**- C; **m**, EDS spectrum showing the constitutive elements in AuNcgs.

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131 Further morphological characterization was performed using Scanning electron microscopy (SEM),
 132 Ultraviolet-visible (UV-Vis) spectroscopy and X-ray powder diffraction (XRD). SEM image shows
 133 well-dispersed, corner truncated AuNcgs with pores in the faces and corners (Fig. 2a). Inverted gray
 134 scale SEM image show transparent white zones that correspond to the holes present in AuNcgs (Fig.
 135 2b). UV-Vis spectra of AuNcgs show the tunability of localized surface plasmon resonance spectra

136 in the near-infrared region, which highly favors the optical properties of the AuNcgs produced (Fig.
 137 2c). The evolution of hollow AuNcgs from solid AgNcbs shifts the localized surface plasmon
 138 resonance peak towards the near-infrared region, which offers several optical properties to
 139 AuNcgs³². XRD spectrum of quick-synthesized AuNcgs (Fig. 2d) was compared with JCPDS data
 140 for pure Au standard (00-004-0784) and AgCl standard (00-031-1238).

Figure 2: : SEM, UV-vis, and XRD characterization of quickly- synthesized AuNcgs. **a**, SEM image of AuNcgs; **b**, inverted- alpha SEM image of AuNcgs; SEM images demonstrates that the AuNcgs synthesized via microwave aided ultrafast method yields nanocages of uniform size and shape, however, certain clumps of nanoparticles were observed that was dispersed upon thorough washing. **c**, UV-vis absorption spectra for AgNcbs and AuNcgs synthesized: AuNcgs-before wash (red), AuNcgs- after wash (blue), AgNcbs (inset), Photograph of AuNcg solution after washing (inset); Upon washing the excess, unbound polymer and impurities of AgCl was washed off, which enhanced the spectral intensity of AuNcgs with a red shift of the peak centre. **d**, XRD spectra of AuNcgs synthesized using microwave oven method showing the peaks (111), (200), (220), (311) and (222). The spectrum (red) represents the peaks arising from AuNcgs and the AgCl impurities present in the mixture of nanocage sample. Based on the spectra, the intensity of (111) is relatively high compared to the (200) peak, showing that the AuNcg has a slight corner truncation, which is evident from the TEM and SEM images as well. Corresponding standard XRD spectra for Au (Blue) and AgCl (Green) were depicted above for comparison. Standard spectra was taken from JCPDS database.

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142 Verification of generation of PA signals

143 The experimental setup to capture PA signals generated by various concentrations of quick-
 144 synthesized AuNcgs is shown in Figure 3a. AuNcg sample solution was serially diluted as
 145 mentioned in the method section and placed in a cuvette before PA signals were acquired. Data
 146 processing was performed and some of the processed signals can be seen in Figure 3b.

147 The mean and standard deviation of the processed UST signal (indicating the strength of PA), at
 148 varying concentrations of the AuNcg solution are plotted against the concentration, as shown in

Figure 3c. The line of best fit with zero-intercept is also plotted. The slope of the line of best fit and R^2 value were determined to be 1.0235 and 0.9938, respectively. The R^2 value of 0.9938 (which is close to 1) indicates that the experimental data are very close to the line of best fit. The gradient of the line of best fit of 1.0235 indicates that the amplitude of the PA waves detected by the UST is directly proportional to the concentration of the AuNcg solution. This is consistent with Eqn. (4) (see **Methods**) and proves that the signals acquired from the experiment were due to the PA waves generated by the AuNcg solution.

Figure 3: PA experiments with AuNcg solution in quartz cuvette. a, Experimental setup. b, PA signals after Hilbert transformation. PA signals of larger amplitude were observed when the concentration of the AuNcg solutions were higher. c, Experimental results for verification of generation of PA signals by AuNcg solution in quartz cuvette. The line of best fit between the PA amplitude and the concentration of AuNcg solution has a gradient of about 1. This is in line with Eqn. (4) in **Methods** and verifies that the quickly-synthesized AuNcgs can produce PA waves and potentially be used as a PA CA.

PAI of varying concentrations of AuNcg solution in tubings

The experimental setup shown in Figure 4a used to acquire PA and ultrasound (US) images of the test samples. In order to illustrate the effect of AuNcg concentration, PA/US images of the separate tubings, each filled with AuNcg solutions of different concentrations, are shown in Figure 4d. In the Figures 4b-c, the left tubing was filled with deionized water, the center tubing was filled with a mixture of deionized water and AuNcg solution (1:1 volumetric ratio) and the right tubing was filled with AuNcg solution alone. The tubings were excited individually for acquiring PA signals. The combined PA/US images can be seen in the Figure 4d.

165 It can be seen from Figure 4d (i) that a weak PA signal was acquired from the top of the left tubing.
166 In Figure 4d (ii), a weak PA signal was also acquired from the top of the center tubing, but two
167 other PA signals were also observed from the top and bottom of the internal section of the tubing.

Figure 4: PAI of varying concentrations of AuNcg solutions in tubings. **a**, PAI experimental setup. **b**, Acrylic holder to hold the three tubings in place. **c**, Close-up of the three tubings. The left tubing was filled with deionized water. It has no AuNcg and the tubing appears to be clear. The center tubing was filled with a mixture of deionized water and AuNcg solution, thus it appears darker compared to the left tubing due to the presence of AuNcgs in the mixture. The right tubing was filled with AuNcg solution. It had the highest concentration of AuNcg solution and appears to be the darkest among the three tubings. **d**, Combined PA/US images when each tubing was separately illuminated for PAI. **(i)**, The left tubing was filled with deionized water and when excited, a weak PA signal can be observed from the tubing. **(ii)**, The center tubing was filled with a mixture of deionized water and AuNcg solution. When excited, two strong PA signals are observed from the internal section of the tubing, which are attributed to the presence of AuNcgs in the solution. **(iii)**, The right tubing was filled with AuNcg solution and when excited, the PA signals are similar to those shown in (ii), but of larger amplitude and more intense. This is attributed to the solution in the right tubing with a higher concentration of AuNcg. These show that the AuNcgs enhance PA signals and the PA enhancement increases with the concentration of the AuNcg solution.

169 The same was observed in Figure 4d (iii), but PA signals from the internal section of the right
170 tubing are observed to be larger in amplitude and more intense. The PA signals from the internal
171 section of the center and right tubings are attributed to the presence of AuNcgs in the solution in the
172 tubings.

173 Two areas are selected from the center tubing where PA signals are observed (top and bottom of
174 tubing's internal section). From the interrogation area of $\sim 0.35 \times 0.35 \text{ mm}^2$, the values are selected
175 and averaged to represent the strength of the PA signals due to the AuNcgs. The same was done for
176 the right tubing. The representative strength of the PA signals from the center tubing to that of the
177 right tubing has a ratio of 0.827:1, which is not proportional to the AuNcg concentration in the
178 tubings of 0.5:1. This could be due to how the PA images are presented by the system, where the
179 algorithms used may not be linear (approximate log compression). However, the trend of a higher
180 AuNcg concentration giving stronger PA signals still holds.

181 **PAI of enucleated porcine eye samples with AuNcg as CA**

182 Figures 5a and 5b shows the image of enucleated porcine eye used for PA imaging and illustrative
183 image of eye, respectively. Two sets of imaging were carried out for each eye sample. The first set
184 of imaging produced a set of PA and US images representing a healthy eye sample (i.e. before the
185 introduction of AuNcgs into the eye), as shown in Figures 5c, BEFORE (i- iv). The second set of
186 imaging was done after the injection (Figs. 5c AFTER (i- iv) of AuNcg solution into the eye, just
187 above the iris.

188 By comparing the schematic diagram of the eye (Fig. 5b) to the combined PA/US images of the eye
189 samples (Figs. 5c), it can be observed that the structural features of the eye are revealed by the US
190 image. The different parts of the eye such as cornea, anterior chamber, lens, iris and posterior pole
191 can be seen. The combined images before and after the injection of AuNcg solution for each eye
192 sample are shown side-by-side (Figs. 5c). It can be observed that PA signals were generated from
193 the iris on the left.

194 An area of $\sim 0.35 \times 0.35 \text{ mm}^2$ was selected as the interrogation region and each set of 50 PA images
195 was considered for the analysis. These areas were selected from the excited iris region where PA
196 signals were produced. The amplitudes of the PA signals within each area were average to represent
197 the strength of the PA signals as presented by the system. PA signals were detected from the
198 pigmented iris before the introduction of AuNcgs, due to the presence of melanin, which is optically
199 absorbing.³³ It was found that after the injection of AuNcg solution into the region above the left iris
200 of the eye samples, the PA signals for eye samples 1 to 4 increased by $46.3 \pm 14.3 \%$, $81.4 \pm 16.7 \%$,
201 $57.9 \pm 14.8 \%$ and $17.6 \pm 17.2 \%$, respectively (Fig. 5d). These results show that the AuNcgs can

202 potentially be used as a PA CA in ocular imaging. Specific targeting of AuNcgs to markers of
 203 diseases can potentially be used to identify diseases³⁰ such as uveal melanoma by using PAI as the
 204 imaging (diagnostic) modality.

Figure 5: PAI of enucleated porcine eye samples with AuNcg. **a**, Injection of AuNcg solution into porcine eye sample. **b**, Schematic diagram of eye. **c**, Combined images of eye samples 1, 2, 3 and 4. Images, 'BEFORE' (i)-(iv) shows the eye samples 1- 4 before injection of AuNcg solution respectively. , Images, 'AFTER' (i)-(iv) shows the eye samples 1- 4 after injection of AuNcg solution respectively, Eye samples before injection showed very little background PA signals due to melanin pigments , however the PA signals measured after the injection of AuNcg solution was drastically increased in intensity. The injected AuNcgs were used to simulate the binding of bio-conjugated AuNcgs on uveal melanoma in the iris region. It can be observed that after the introduction of AuNcg, the strength of the PA signals in the iris increases and becomes more intense. This demonstrates that the quickly-synthesized AuNcgs can potentially be used to create contrast enhancement in PA images for ocular disease diagnosis. **d**, Increase in strength of PA signals after injection of AuNcg solution. After the introduction of AuNcg solution into the eye, the amplitude of the PA signals in eye samples 1, 2, 3 and 4 increased by 46.3%, 81.4%, 57.9% and 17.6%, respectively. The average increase in the amplitude of the PA signal is about 50.8%.

205

206 3. Conclusions

207 Highly efficient AuNcgs were synthesized using microwave oven heating technology as an ultra-
 208 fast method in the production of AuNcgs.²² Quickly-synthesized AuNcgs were characterized using
 209 HRTEM, SEM, XRD and UV-Vis spectroscopy and were found uniform in size and shape. It was
 210 observed that the UV-Vis absorption spectra of quickly-synthesized AuNcgs was spanning across

the near-infrared region, which is highly beneficial for several optoelectronics and high contrast imaging applications. Here, we have demonstrated a detailed instrumentation for the PA experiments performed using the quickly-synthesized AuNcgs produced via microwave heating technology for the first time. Preliminary studies were conducted to determine the ability of quickly-synthesized AuNcgs to generate PA signals similar to conventionally synthesized and reported AuNcgs.²⁴ This study was conducted using different concentrations of the quickly-synthesized AuNcg solutions. The results show that the strength of the PA signals is directly proportional to the concentration of the AuNcg solution and verified that PA signals were generated by the AuNcgs under pulsed optical excitation. In the subsequent study, high contrast PA images were captured with varying concentrations of AuNcgs in separate tubings. A commercial US imaging scanner, integrated with a tunable nanosecond pulsed laser was used as the imaging platform; it has a UST set within a clinical-style imaging probe for convenient handling. The acquired PA images showed that tubings with higher concentrations of AuNcg solutions will result in the detection of stronger PA signals. The source of the PA signals can also be compared by referring to its corresponding US images, which provide structural information of the imaged region. AuNcgs were used as PA CA for the first time in ocular imaging. PA and US images were acquired from the enucleated porcine eye models and was followed by the injection of the quickly-synthesized AuNcgs into the eye models to simulate uveal melanoma condition. Another set of PA and US images were acquired and compared to the set of images acquired prior to the injection of AuNcgs. The results show that the approximately 50.8 % increase in the strength of the PA signals (increased by 17.6 % to 81.4 %) was observed after AuNcgs were introduced into the eye models. This demonstrates that the quickly-synthesized AuNcgs can potentially be used to create contrast enhancement in PA images for ocular disease diagnosis. Uveal melanoma is a type of intraocular cancer that is generally found in the iris, choroid, and ciliary body. An early diagnosis of this condition can help the patient from developing painful eye condition leading to vision loss and in certain cases of deaths due to metastasis^{29,30}. It was reported that bioconjugated AuNcgs were demonstrated for its use as CAs in the cancer cell imaging.³⁰ It is envisaged that, based on the analysis presented in this manuscript, quickly-synthesized AuNcgs can be used as a high strength PA CA to potentially diagnose uveal melanoma. A future improvement to the hybrid-modality imaging system is to integrate the laser excitation with clinical-style imaging probe. This removes the need to adjust the position of the laser excitation when the imaging probe is moved to image another region. Integrating the PA and US images of the eye models makes hybrid-modality imaging possible, to form combined PA/US images to provide complementary structural and optical absorption-based information. This allows the source of the PA signals to be known by referring to the structural information provided by the US images. Using the AuNcgs as markers of uveal

246 melanoma in hybrid-modality imaging can help to determine the size (spread and depth) and
247 location of uveal melanoma relative to other ocular structures. This can help clinicians make better
248 diagnostic evaluation and confirmation of diseases.

249 **4. Experimental Section**

250 **Chemicals and instruments**

251 Polyvinylpyrrolidone ($M_w \approx 55,000$) (Cat.#856568), sodium sulfide nonahydrate (Cat.#208043) and
252 gold (III) chloride trihydrate (Cat.#G4022-1G) were procured from Sigma-Aldrich, Japan. Ethylene
253 glycol (Cat.#14114-00) and silver nitrate (Cat.#37075-30) were purchased from Kanto chemicals,
254 Japan. All other chemicals and reagents used were of analytical grade.

255 AuNcgs were characterized using Scanning Electron Microscope (SEM), High Resolution
256 Microscope-Transmission Electron Microscope (HRTEM), X-ray powder diffraction (XRD) and
257 Energy-Dispersive X-Ray Spectroscopy (EDS). SEM images were taken using Hitachi, SU6600.
258 TEM images were recorded with JEOL, HRTEM (JEM-ARM200F, 200 kV) fitted with EDS (JEOL,
259 DrySD100GV). UV-Vis absorption spectra of as-synthesized AgNcbs and AuNcgs were measured
260 with Beckman Coulter Spectrophotometer (DU730). XRD spectra were recorded using RIGAKU
261 X-ray diffractometer (SmartLab9kW) and data was compared with Joint Committee on Powder
262 Diffraction Standards (JCPDS) database.

263 PAI experiments were conducted using following instruments: Nanosecond pulsed laser (Vibrant
264 355 II, Opotek Inc.), Power meter (Nova Display and 12A-V1, Ophir), Beamsplitter (BSW10,
265 Thorlabs), Neutral density filter (ND30A, Thorlabs), Objective lens 1 (UPlan FLN 10x, Olympus),
266 Objective lens 2 (MPlan N 5x, Olympus), Photodiode (SM05PD2B, Thorlabs), digitizer (200 MS/s,
267 Razor CompuScope 1622, GaGe), 3-axis stage (T-LS28M, Zaber Technologies), UST (V110-RM,
268 Olympus Panametrics-NDT), Pre-amplifier (5662, Olympus Panametrics-NDT) and US imaging
269 scanner (UltraVision 64B Research Platform, Winprobe) with clinical-style imaging probe (L8,
270 Winprobe).

271 **Microwave mediated synthesis of AuNcgs**

272 AgNcbs were synthesized based on the microwave assisted polyol method mentioned elsewhere.²⁸
273 Briefly, 10 ml of ethylene glycol (EG) solution of 250 μ M Na₂S was vigorously stirred after adding
274 0.075 M PVP. The mixture was injected drop wise using a syringe into a 10 ml of EG solution of
275 AgNO₃ (0.05 M) under constant magnetic stirring. On addition, the solution turned wine colored
276 due to the formation of Ag₂S. Wine colored solution was intermittently heated (stop and start

method) in a microwave and swirled manually for thorough mixing between the heating steps. Heating experiment was conducted in a microwave oven (YJ-50H8, LG Electric, Japan) operating at frequency of 50 Hz, power consumption of 1000 W and rated high frequency output of 500 W. Khaki-colored solution containing AgNcbs were formed within seconds of reaction. Nanocubes were washed several times before galvanic replacement with HAuCl₄ solution. Galvanic replacement reaction was conducted using 5 ml of 0.1 mM HAuCl₄ solution with 550 µl of as-synthesized AgNcbs in microwave oven.²² AgNcb solution was first mixed with 5 ml of 9 mM aqueous solution of PVP before introducing Au solution. The mixture was heated in the microwave oven intermittently until a stable pale-purple color was obtained. AuNcgs were washed several times with water and ethanol/water 1:1 mixture by volume and dispersed in deionized water for characterization and PAI studies.

Concentration of the AuNcg solutions was measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), based on the quantification of Au (III) ions present in various test AuNcg solutions. ICP-MS measurement was performed using iCAP-Q, Thermo Scientific with a plasma Ar flow rate of 15 L/min and a dwell time of 100 ms for each sample. All the samples were microwave (ETHOS UP) digested using 5% aqua regia. A series of Au standard solution were prepared and measured for obtaining a calibration curve, using which the Au (III) concentrations in the test samples were found.

Experimental setup for the verification of generation of PA signals by AuNcg solution in cuvette

The AuNcg reported in this paper was suspended in deionized water. In order to prove that the AuNcg solution was able to produce PA signals, an experiment measuring AuNcg solutions of varying concentrations was conducted. The optical absorption coefficient of a solution is dependent on its concentration *Conc* and molar absorption ε_α , as seen in Eqn. (2). Substituting Eqn. (2) into Eqn. (1) gives Eqn. (3). Considering that only an excitation wavelength λ is used, the Grüneisen parameter Γ and ε_α are constants and the measurements are corrected for fluence *F* fluctuations, Eqn. (3) is reduced to Eqn. (4). Equation (4) shows that if the AuNcg is able to produce PA signals, the strength of the PA signals is directly proportional to its concentration.

$$\mu_\alpha(\lambda) = Conc \cdot \varepsilon_\alpha(\lambda), \quad (2)$$

$$P_0(\lambda) = \Gamma F(\lambda) Conc \cdot \varepsilon_\alpha(\lambda), \quad (3)$$

$$P_0 \propto Conc, \quad (4)$$

308 The experimental setup to capture the PA signals for this purpose consisted of a tunable nanosecond
309 pulsed laser at 800 nm with a frequency of 10 Hz (Vibrant 355 II, Opotek Inc.) to provide the
310 required optical excitation for PA measurement. The pulse energy was measured to be $\sim 26 \mu\text{J}$ using
311 a power meter (Nova Display and 12A-V1, Ophir). When the pulse reached the beamsplitter
312 (BSW10, Thorlabs), the reflected light was directed towards the neutral density filter (ND30A,
313 Thorlabs) and focused by objective lens 1 (UPlan FLN 10x, Olympus) onto the photodiode
314 (SM05PD2B, Thorlabs). The pulse detected by the photodiode triggered the digitizer (200 MS/s,
315 Razor CompuScope 1622, GaGe). The transmitted light through the beamsplitter travelled towards
316 objective lens 2 (MPlan N 5x, Olympus) before reaching the AuNcg solution placed in a quartz
317 cuvette (104-10-40, Hellma Analytics), which was held in place by a 3-axis stage (T-LS28M, Zaber
318 Technologies). Upon excitation, the PA wave was detected by the UST (V110-RM, Olympus
319 Panametrics-NDT), which was in direct contact with the quartz cuvette. A pre-amplifier (5662,
320 Olympus Panametrics-NDT) with a 54 dB gain amplified the detected signal before being directed
321 to the digitizer. When the digitizer was triggered, the signals from both the photodiode measuring
322 the fluence of the pulsed excitation and UST measuring the strength of the PA waves were acquired.
323 The signals were averaged over 100 pulses for each measurement to improve the signal-to-noise
324 ratio. A custom developed LabView® software was used to control the laser, 3-axis stage, digitizer,
325 and to save the averaged data. 5 measurements were acquired for each concentration of AuNcg
326 solution.

327 The quartz cuvette was initially filled with 1.5 ml of the AuNcg solution using a single-channel
328 pipettor (4075, Lambda™ Plus Corning). The initial concentration of the AuNcg solution was 100%
329 and with this concentration, measurements were carried out for 5 times. This is continued for a total
330 of 9 concentrations: 100%, 80%, 64%, 51.2%, 40.96%, 32.77%, 26.21%, 20.97% and 16.78%. A
331 control experiment was also carried out with 0% AuNcg concentration using only deionized water.
332 A total of 50 measurements (5 for each of 10 concentrations) were acquired.

333 **Experimental setup for PAI of varying concentrations of AuNcg solution in tubings**

334 PA and US images of the tubings filled with solutions of different AuNcg concentrations were
335 acquired using a commercial US imaging scanner (UltraVision 64B Research Platform, Winprobe)
336 which was integrated with a tunable nanosecond pulsed laser (Vibrant 355II, Opotek Inc). The
337 experimental setup is shown in Figure 4a. This reported integrated system³ enabled hybrid-modality
338 imaging where PA and US images can both be acquired to provide combined images of
339 complementary structural and optical absorption-based information. The images were acquired
340 using a 128-element linear array UST set within a clinical-style imaging probe (5-10 MHz, L8,
341 Winprobe). The elements had a pitch of 0.3 mm, thus the UST had an azimuthal length of 38.4 mm

342 for US imaging. For PAI, the azimuthal length was only 19.2 mm as only the center 64 elements in
343 the UST were used. The UST produced US pulses and the detected echoes formed US images. The
344 imaging scanner formed multiple US images and waited for the PA trigger from the laser. The PA
345 trigger cause the scanner to stop producing US pulses and the detected UST signals were used to
346 form a PA image. Thereafter, the UST produced US pulses and USI resumed. This process was
347 repeated and PA and US images were produced at 10 Hz and ~40 Hz, respectively.

348 Three transparent flexible plastic tubings (S3 E-3603, Tygon) were used in this experiment. They
349 have inner and outer diameters of 3.2 mm and 4.8 mm, respectively. They were held in place from
350 the ends and placed at ~7.5 mm apart using an acrylic holder (Fig. 4b). The left tubing was filled
351 with deionized water, the center tubing was filled with a mixture of deionized water and AuNcg
352 solution (1:1 volumetric ratio) and the right tubing was filled with AuNcg solution only (Fig. 4c).
353 They were then left to settle for ~30 minutes.

354 During the experiment, the tubings were partially submerged in the water and the linear-array UST
355 was placed transverse to and above the lowermost point of the three tubings. PA excitation was
356 delivered to one tubing at a time. This helped to ensure that constant excitation fluence was
357 delivered to each tubing. In order to get PA signals in this experiment, the excitation beam with
358 wavelength of 500 nm and diameter of 5 mm was used. The pulse energy was measured to be ~590
359 μ J using a power meter (Nova Display and 12A-V1, Ophir). First, the left tubing which was placed
360 below the UST was excited and PA images were taken. This is followed by exciting the center and
361 right tubings to obtain the relevant PA images. 50 PA and 50 US images were captured when each
362 tubing was imaged.

363 **Experimental setup for PAI of enucleated porcine eye samples with AuNcg as CA**

364 Biological PA and US images of enucleated porcine eye samples were also acquired using the same
365 system as mentioned in the above section. The eye samples were held in place by a holder and
366 aligned to face upwards. The eyes were positioned towards the right of the UST so that the left iris
367 appeared at the center of the images. The UST was placed ~2 cm above the lens of the eye.

368 Two sets of imaging were conducted by taking 50 PA and 50 US images of the porcine eye samples.
369 In this study, the laser beam with wavelength of 500 nm and diameter of 3 mm was used for
370 excitation at the iris. The pulse energy was measured to be ~71 μ J using a power meter (Nova
371 Display and 12A-V1, Ophir). The first imaging produced a set of PA and US images, which
372 represents that of a healthy eye sample. It was then removed from the water tank and ~0.15 ml of
373 AuNcg solution was slowly injected into the eye, just above the iris on the left (Fig. 6A). The eye
374 was then left untouched for ~20 minutes so that the AuNcg solution can settle. These simulated

375 samples, prepared following the above procedure represent uveal melanoma, which is tagged by
376 bio-conjugated AuNcgs.

377 **Preparation of porcine eye samples**

378 Four porcine eye samples (*Sus scrofa domestica*) were used in this study. The porcine eye samples
379 with extraocular tissues removed were acquired from local abattoir. The samples were placed on ice
380 until the experiments began. Samples that were found to be free from any signs of deterioration
381 after visual inspection were used for experiment. The eye samples were used within 6 hours of
382 death. The study was conducted according to NTU's regulations on biosafety and the regulations of
383 Agri-Food & Veterinary Authority of Singapore.

384 **PA and US data processing**

385 **Verifying generation of PA signals by AuNcg solution in cuvette:** Data acquired from the
386 experiments were processed offline using in-house written script in MATLAB®. Data processing
387 started with Hilbert transformation of the UST signals and correction for fluence fluctuations using
388 signals from the photodiode (Fig. 3b). Hilbert transformation is widely used in analytical signal
389 analysis to pick up the envelopes of vibration signals.³⁴ These signals were corrected for any
390 background PA signals using the mean value of the signal from the sample with 0% AuNcg
391 concentration. The mean and standard deviation of the measurements of the concentrations were
392 calculated and normalized.

393 **PAI of AuNcg solution in tubings and porcine eye samples:** Data acquired from the experiments
394 were processed offline using in-house written script in MATLAB®. Each set of 50 PA and 50 US
395 images were averaged to form a representative image with reduced random noises. Signals of weak
396 intensity in the representative PA images were also removed for better clarity. For each eye sample,
397 the two representative PA images were normalized with respect to the strongest signal in the PA
398 image before the AuNcg solution was introduced.

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406 **Conflict of Interest**

407 The authors declare no conflict of interest

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460 AUTHOR CONTRIBUTIONS

461 S.R., T.M., M.V.M., and D.S.K. conceived the idea and designed the project. S.R. and H.T.L.
462 conducted the experiments and imaging studies, respectively. S.R., H.T.L., M.V.M. and D.S.K.
463 analyzed the data. S.R., H.T.L., M.V.M. and D. S. K. wrote the paper.

464

465 FIGURE LEGENDS

466 **Figure 1: HRTEM characterization of quickly- synthesized AuNcgs.** **a**, HRTEM image of
467 AuNcg; Corresponding FFT image for the AuNcg (Inset); **b**, HRTEM image of the corner of AuNcg
468 shown in figure **a**; **c**, Line profile of FFT image shown in figure **a**-inset; **d**, Line profile from the
469 highlighted area of the HRTEM image of AuNcg shown in figure **b**; **e**, low magnification TEM
470 image of AuNcgs in cluster; **f**, High magnification bright field TEM image of AuNcg ; **g**, HAADF-
471 STEM image of AuNcg shown in **f**; **h- k**, EDS mapping images for AuNcg: **h**- Bright field; **i**- Au; **j**-
472 Ag; **k**- C; **m**, EDS spectrum showing the constitutive elements in AuNcgs.

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Figure 2: : SEM, UV-vis, and XRD characterization of quickly- synthesized AuNcgs. **a**, SEM image of AuNcgs; **b**, inverted- alpha SEM image of AuNcgs; SEM images demonstrates that the AuNcgs synthesized via microwave aided ultrafast method yields nanocages of uniform size and shape, however, certain clumps of nanoparticles were observed that was dispersed upon thorough washing. **c**, UV-vis absorption spectra for AgNcbs and AuNcgs synthesized: AuNcgs-before wash (red), AuNcgs- after wash (blue), AgNcbs (inset), Photograph of AuNcg solution after washing (inset); Upon washing the excess, unbound polymer and impurities of AgCl was washed off, which enhanced the spectral intensity of AuNcgs with a red shift of the peak centre. **d**, XRD spectra of AuNcgs synthesized using microwave oven method showing the peaks (111), (200), (220), (311) and (222). The spectrum (red) represents the peaks arising from AuNcgs and the AgCl impurities present in the mixture of nanocage sample. Based on the spectra, the intensity of (111) is relatively high compared to the (200) peak, showing that the AuNcg has a slight corner truncation, which is evident from the TEM and SEM images as well. Corresponding standard XRD spectra for Au (Blue) and AgCl (Green) were depicted above for comparison. Standard spectra was taken from JCPDS database.

Figure 3: PA experiments with AuNcg solution in quartz cuvette. **a**, Experimental setup. **b**, PA signals after Hilbert transformation. PA signals of larger amplitude were observed when the concentration of the AuNcg solutions were higher. **c**, Experimental results for verification of generation of PA signals by AuNcg solution in quartz cuvette. The line of best fit between the PA amplitude and the concentration of AuNcg solution has a gradient of about 1. This is in line with Eqn. (4) in **Methods** and verifies that the quickly-synthesized AuNcgs can produce PA waves and potentially be used as a PA CA.

Figure 4: PAI of varying concentrations of AuNcg solutions in tubings. **a**, PAI experimental setup. **b**, Acrylic holder to hold the three tubings in place. **c**, Close-up of the three tubings. The left tubing was filled with deionized water. It has no AuNcg and the tubing appears to be clear. The center tubing was filled with a mixture of deionized water and AuNcg solution, thus it appears darker compared to the left tubing due to the presence of AuNcgs in the mixture. The right tubing was filled with AuNcg solution. It had the highest concentration of AuNcg solution and appears to be the darkest among the three tubings. **d**, Combined PA/US images when each tubing was separately illuminated for PAI. **(i)**, The left tubing was filled with deionized water and when excited, a weak PA signal can be observed from the tubing. **(ii)**, The center tubing was filled with a mixture of deionized water and AuNcg solution. When excited, two strong PA signals are observed from the internal section of the tubing, which are attributed to the presence of AuNcgs in the

509 solution. (iii), The right tubing was filled with AuNcg solution and when excited, the PA signals are
510 similar to those shown in (ii), but of larger amplitude and more intense. This is attributed to the
511 solution in the right tubing with a higher concentration of AuNcg. These show that the AuNcgs
512 enhance PA signals and the PA enhancement increases with the concentration of the AuNcg
513 solution.

514

515 **Figure 5: PAI of enucleated porcine eye samples with AuNcg. a,** Injection of AuNcg solution
516 into porcine eye sample. **b,** Schematic diagram of eye. **c,** Combined images of eye samples 1, 2, 3
517 and 4. Images, 'BEFORE (i)- (iv)' shows the eye samples 1- 4 before injection of AuNcg solution
518 respectively. Images, 'AFTER (i)- (iv)' shows the eye samples 1- 4 after injection of AuNcg
519 solution respectively, Eye samples before injection showed very little background PA signals due to
520 melanin pigments, however the PA signals measured after the injection of AuNcg solution was
521 drastically increased in intensity. The injected AuNcgs were used to simulate the binding of bio-
522 conjugated AuNcgs on uveal melanoma in the iris region. It can be observed that after the
523 introduction of AuNcg, the strength of the PA signals in the iris increases and becomes more
524 intense. This demonstrates that the quickly-synthesized AuNcgs can potentially be used to create
525 contrast enhancement in PA images for ocular disease diagnosis. **d,** Increase in strength of PA
526 signals after injection of AuNcg solution. After the introduction of AuNcg solution into the eye, the
527 amplitude of the PA signals in eye samples 1, 2, 3 and 4 increased by 46.3%, 81.4%, 57.9% and
528 17.6%, respectively. The average increase in the amplitude of the PA signal is about 50.8%.

529

530