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It is essential to monitor and understand the diseased tissue before starting the treatments. Recently, many efforts have been devoted to explore theranostic agents that encapsulate therapeutic agents and diagnostic probes into one package. However, these physically mixed two agents may slowly dissociate from the carrier at different rates during the circulation in blood, leading to quite different biodistribution and pharmacokinetics. Thus, it is important to explore a photothermal agent which itself can serve as a contrast agent. Though some inorganic dual functional theranostic agents such as gold nanoparticles have been explored, most of them still suffer from poor biodegradability and biocompatibility. Herein, we report a theranostic agent based on a narrow-bandgap small molecule, benzo[1,2-c;4,5-c']bis[1,2,5]thiadiazole-4,7-bis(9,9-dioctyl-9H-fluoren-2-yl)thiophene (denoted as BBT-2FT), with strong absorption of near infrared (NIR) light. Colloidal nanoparticles composed of BBT-2FT show photoacoustic signal intensity 10 times higher than that of blood, and high photothermal conversion efficiency ($\eta = 40\%$) under irradiation of 800-nm laser light that kills over 90% HeLa cells in 10 mins.

Introduction

Theranostic, defined as a material that combines the modalities of therapy and diagnosis, has attracted great attention since it was coined in 2002¹⁻³. Before starting treatments to various diseases such as cancer, it is important to monitor and understand the status and location of the disease. In contrast to using two separate agents, theranostic aims to incorporate various agents into one material, in order to realize imaging and therapy simultaneously. For instance, some of the therapeutic strategies developed for different types of cancer treatments, such as nucleic therapy, chemotherapy, photothermal therapy, photodynamic therapy, and radiation therapy, can be functionalized with imaging strategies, such as magnetic resonance imaging (MRI), nuclear imaging (PET/CT), photoacoustic imaging, and fluorescence imaging⁴⁻¹⁰.

Among a variety of diagnosis techniques, photoacoustic tomography (PAT) is a promising medical imaging technique that combines rich optical contrast and scalable high ultrasonic resolution in a single modality¹¹⁻¹⁴. PAT can provide deeper

tissue imaging than other pure optical imaging modalities (Fluorescence microscopy, Raman microscopy, Optical coherence tomography, etc.). In PAT, short (nanosecond) non-ionising laser pulses are absorbed by the biological tissue leading to transient thermal expansion of the tissue and subsequent ultrasound emission. The generated ultrasound waves are acquired by ultrasound transducer to form a photoacoustic image. PAT has been proven to be a promising technique for imaging biological features from organelle to organs¹⁴. The application of PAT includes, but not limited to, small animal brain imaging, breast cancer imaging, monitoring of vascularisation, tumor angiogenesis, blood oxygenation, total haemoglobin concentration, etc.¹⁵⁻²¹

Biological tissues have relatively low absorption in the near infrared (NIR) region^{22, 23}. Therefore, NIR light has been used for deep tissue PAT imaging. Several exogenous contrast agents with high absorption in the NIR region have been synthesized and used to enhance the contrast for deep tissue PAT imaging²⁴. Noble metallic nanoparticles with different shapes such as gold (Au) nanoshells²⁵, nanorods²⁶, and nanocages^{27, 28}, nanobeacons^{29, 30} have been widely used as contrast agents for PAT imaging. Other inorganic NPs, such as single-walled carbon nanotubes (SWCNTs)^{31, 32} and copper sulphide (CS) NPs³³, served as good contrast agents for deep PAT imaging. Quantum dots were also used as multi-modal contrast agent in photoacoustic and photothermal imaging³⁴.

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Nevertheless, the biodegradability and long-term toxicity of these inorganic materials remain an issue for their use in clinical trials. Organic dyes such as IRDye-800, and indocyanine green (ICG)^{35, 36} have better biocompatibility and drug-delivery capability. But they suffer from relatively small optical absorption cross-section, and could be easily removed by the renal system due to their relatively small size (<10 nm). Dye-doped porphyrin organic NPs have been reported for PAT imaging³⁷, but their relatively large diameter (>100 nm) could result in clearance by macrophage system mostly by the liver and spleen. Recently, biocompatible polypyrrole organic NPs (~46 nm in diameter) have been demonstrated for deep tissue imaging in PAT system³⁸.

Among many strategies for cancer treatment, photothermal therapy has been widely used due to its advantages such as high specificity, minimal invasiveness, low toxicity to normal tissues, and excellent anti-cancer efficacy.³⁹⁻⁴¹ As a consequence, many efforts have been devoted to explore various theranostic nanomaterials, especially combining NIR photoacoustic imaging and photothermal therapy due to the deep penetration of tissues, high specificity, minimal invasiveness, selective damage and excellent anti-cancer efficacy. Though many theranostic platforms⁴²⁻⁴⁶ have been established as the combining form of the therapeutic agents and imaging agents, the two agents may slowly dissociate from the carrier at different rates during the circulation in blood, leading to quite different biodistribution and pharmacokinetics. Thus, it is important to explore a photothermal agent, which can serve as a contrast agent itself. To that end, it is necessary to incorporate photoacoustic imaging and photothermal therapy into one agent to realize diagnosis and therapy together. Recently, some dual-modal theranostic materials, such as combining photoacoustic imaging and PTT using inorganic nanoparticles (NPs)^{34, 47-51} and conjugated polymers⁵² have been reported.

Benzo[1,2-*c*;4,5-*c'*]bis[1,2,5]thiadiazole (BBT) based derivatives are well-known narrow-bandgap building blocks for organic optoelectronic devices⁵³⁻⁵⁹. Taking the advantage of the strong light absorption of BBT derivatives in near NIR window that can benefit deep tissue imaging and therapy, Wang and coworkers⁶⁰ recently reported that colloidal nanoparticles composed of a small molecular BBT derivative (denoted as BBTEHT) showed high photothermal conversion efficiency and robust photostability compared to gold NPs^{61, 62} for effective treatment of cancer cells. The advantages of these small-molecular agents over those inorganic and polymeric analogues include their well-defined molecular structure, good synthetic reproducibility, and potentially better biodegradability. We expected that the impressive photothermal performance of BBT derivatives would also enable their applications as contrast agents for photoacoustic imaging, leading to a new type of theranostic agent that integrate the functionality of both photothermal therapy and photoacoustic imaging.

Herein, we report such a theranostic agent based on a small-molecular BBT derivative, benzo[1,2-*c*;4,5-

c']bis[1,2,5]thiadiazole-4,7-bis(9,9-dioctyl-9*H*-fluoren-2-yl)thiophene (denoted as BBT-2FT). BBT unit, due to its hypervalent sulfur atoms stabilized in quinoidal structures in a conjugated backbone, is a strong electron accepting unit that ensures strong NIR absorption of BBT-2FT in the therapeutic optical window. The biradical nature of BBT unit as well as the presence of thermally accessible triplet states may also contribute to the NIR absorption.⁶³ The BBT-2FT NPs prepared through nanoprecipitation in the presence of block polymer PEG-*b*-PCL possess excellent stability and biocompatibility. The NPs not only demonstrated significant photothermal conversion under 808 nm laser irradiation, but also served as photoacoustic imaging contrast agent and provided strong photoacoustic signal compared to blood. With the treatments of BBT-2FT agents and laser, 90% HeLa cells could be killed in 10 min.

Results and discussion

The preparation and characterization of BBT-2FT NPs

Scheme 1. Schematic illustration of the preparation of BBT-2FT nanoparticles and their applications in NIR photothermal therapy and photoacoustic imaging.

Scheme 1 shows the molecular structure of BBT-2FT and a schematic presentation of the NPs formed through a nanoprecipitation process. Narrow-band gap molecule BBT-2FT was synthesized following a procedure described in the SI, which was also confirmed by ¹H-NMR (Figure. S2), ¹³C-NMR, and MALDI-TOF mass spectroscopy (Figure. S1). BBT-2FT showed two absorption peaks in an organic solvent such as THF, one around at 450 nm with small shoulder and the other at 880 nm, with an onset around 1300 nm. The molar absorption coefficient of BBT-2FT in toluene at 808 nm was calculated to be $2.7 \times 10^4 \text{ L} \cdot \text{Mol}^{-1} \cdot \text{cm}^{-1}$.

In this study, we chose an amphiphilic block polymer PEG-*b*-PCL instead of Pluronic F127 as the stabilizer for the preparation of BBT-2FT colloidal NPs, as some agglomeration of Pluronic F127 micelles loaded with BBT-EHT was observed under NIR laser light irradiation.⁶⁰ Such intermicellar

agglomeration can be attributed the relatively low lower critical solution temperature (LCST) of Pluronic F127 compared to that of PEG-*b*-PCL. As a result, Pluronic F127 forms gels in water over a much broader temperature range than PEG-*b*-PCL-*b*-PEG.^{64, 65} These results suggest that PEG-*b*-PCL micelles have better photothermal stability than Pluronic F127 micelles, which can benefit their applications in bioimaging and photothermal therapy. PEG-*b*-PCL was synthesized via ring-opening polymerization of β -caprolactone using monomethyl-PEG_{5k} as the initiator. The number average molecular weight (M_n) calculated by GPC was 11000. The molar ratio of hydrophilic PEG chain to hydrophobic PCL chain is 1:1, which is consistent with the result calculated by ¹H-NMR. Details about the synthesis and characterization of PEG-*b*-PCL are presented in Supporting Information.

Two methods were tested to prepare the colloidal NPs of BBT-2FT in presence of PEG-*b*-PCL (critical micelle concentration = 5 mg mL⁻¹ in water)⁶⁶ that not only ensures good biocompatibility, but also enhances colloidal stability of BBT-2FT NPs in water. The experimental details about the preparation of the NPs are described in Supporting Information. Briefly, in Method A, the mixture of BBT-2FT and PEG-*b*-PCL in THF/water (1/10, by volume) was subjected to dialysis against water to remove THF. In Method B, THF in the mixture was removed by evaporation in air under vigorous stirring at room temperature. In both cases, the self-assembly was driven by the hydrophobic interaction between the PCL and BBT-2FT which collapsed to form the core of the NPs, while the hydrophilic PEG forming the shell of the NPs provided colloidal stability.

PEG-*b*-PCL micelles before and after being loaded with BBT-2FT were characterized by both transmission electron microscopy (TEM) and dynamic light scattering (DLS). A representative TEM image of the BBT-2FT-loaded PEG-*b*-PCL micelles prepared by Method A is shown in Figure 1a. Most of the NPs appear spherical with different sizes, while a minor population of tadpole-like particles (labeled by circles in Figure 1a) were also observed. The average diameter of these NPs measured by TEM is 46 ± 11 nm, which is smaller than that (68 nm) measured by DLS in the hydrated state. Both TEM and DLS results showed that the average size of the BBT-2FT-loaded PEG-*b*-PCL micelles was slightly larger than that of PEG-*b*-PCL blank micelles (Figure S7) prepared by the same method.

Compared to the NPs prepared by Method A, those prepared by Method B appeared more uniform under TEM (Figure S9a-b), although the particle size is generally larger. The BBT-2FT-loaded NPs had an average diameter of 81 ± 13 nm, which is slightly larger than that (76 ± 16 nm) of PEG-*b*-PCL blank micelles. The hydrodynamic diameter of the hydrated micelles in water measured by DLS also increased from 90 to 100 nm after being loaded with BBT-2FT. These micellar particles with suitable sizes are expected to target cancer tissues through enhanced permeation retention (EPR) effect and to prevent a blockage of blood vessels or being eliminated by the body's reticuloendothelial system (RES). Most of the experiments below involved BBT-2FT NPs prepared through Method A unless noted specifically.

BBT-2FT shows a strong NIR absorption peak at 880 nm, which is broader compared to that of commercial ICG

Figure.1 (a) TEM image of BBT-2FT NPs from dispersion of 50 $\mu\text{g mL}^{-1}$. (b) UV-Vis-NIR spectra of BBT-2FT NPs dispersed in water and ICG water solution. (c) Temperature change plots of different concentrations of BBT-2FT NPs upon irradiation by an 808 nm laser with a power density if 1.77 W/cm² for 10 min. (d) Temperature elevation of BBT-2FT NPs over six laser ON/OFF cycles of NIR laser irradiation. All of the NPs presented in this figure were prepared through Method A.

molecules. The photothermal effect induced by NIR laser illumination at 808 nm with a power density of 1.77 W cm⁻² for 10 min in the presence of BBT-2FT NPs was investigated by monitoring the temperature of 1 mL aqueous dispersion of BBT-2FT NPs at various concentrations (25, 50, and 100 $\mu\text{g mL}^{-1}$). Obvious concentration-dependence was observed under laser irradiation of the aqueous dispersions containing BBT-2FT NPs, whereas pure water as a control showed little change in temperature under the same conditions of laser irradiation. We measured the photothermal conversion efficiency following the method reported previously (Supporting information).⁶⁷ The η value was calculated to be 47%, which is higher than that of the BBT small molecule that we reported previously⁶⁰ and those of other reported photothermal agents such as polypyrrole ($\eta = 40\%$) and Au nanorods ($\eta = 22\%$).⁶⁷

To further investigate the photostability of BBT-2FT NPs, six cycles of ON/OFF NIR laser irradiation were conducted. The continuous laser (808 nm, 1.77 W/cm²) instead of high power pulse laser was used to test the photothermal effect, since the pulse laser used in photoacoustic system causes the temperature increasing too fast to record. We note that the continuous laser was also used in the cell experiments described later. Dispersion of BBT-2FT NPs (50 $\mu\text{g mL}^{-1}$) was irradiated with NIR laser for 10 min (Laser ON, Figure. 1d), followed by naturally cooling (without laser irradiation) to room temperature over 10 min (Laser OFF). This cycle was repeated six times in order to investigate the photostability of BBT-2FT NPs. The recorded temperature change indicated no significant photoinduced degradation of BBT-2FT NPs under

the present experimental conditions (Figure. 1d). Nevertheless, the TEM images (Figure S9 c-d) showed some morphological changes of the nanoparticles (prepared via Method B) after

Figure. 2 Schematic diagram of the PLD-PAT imaging system. PLD: Pulsed laser diode, GG: Ground glass, S: Sample, M: Motor, LD: Laser driver, R/A/F: Receiver, amplifier and filter unit, DAQ: Data acquisition card, UST: Ultrasound transducer.

laser irradiation. For example, the overall size distribution of the micelles appeared broader (87 ± 19 nm by TEM) after 6 cycles of ON/OFF laser treatment, and some polyhedral nanoparticles were observed. Such changes might be caused by the photothermal annealing effect that induced the local crystallization of the PCL chains within the cores of the micelles. Nevertheless, the morphological and size changes observed under TEM could not be detected by dynamic light scattering (Figure S9 e), suggesting that the average size and colloidal stability of the nanoparticles were not significantly affected by the laser irradiation.

The promising photothermal conversion efficiency of BBT-2FT NPs prompted us to investigate the application of these NPs for photoacoustic imaging as well as photothermal therapy, as discussed below.

PAT imaging system

The PAT imaging system used in the current study is depicted in Figure. 2. The pulsed laser diode (PLD) (Quantel, France) provides ~ 136 ns pulses at a wavelength of ~ 803 nm and pulse energy of ~ 1.4 mJ at maximum 7 kHz repetition rate. A ground glass (GG) is used to make the laser beam more homogeneous. The sample and the transducer were immersed in water for coupling of PA signal to the ultrasound transducer (UST). The photoacoustic (PA) signal generated by the sample was received by a non-focus transducer (V323-SU/2.25 MHz, Olympus NDT) with 13 mm active area and $\sim 70\%$ nominal bandwidth. The UST was driven by a computer-controlled

stepper motor (M) to move continuously in a circular geometry. The signals are subsequently amplified, and band pass filtered by ultrasound signal receiver/amplifier/filter (R/A/F) unit (Olympus-NDT, 5072PR), and then digitized and recorded by the PC with data acquisition card (DAQ) (GaGe, compuscope 4227) installed in it. Usually, low-frequency ultrasound detectors (1-5 MHz) are used in PAT, so the DAQ card was operated at a sampling frequency of 25 Ms/s. Finally, the computer collected PA signals were used to reconstruct the PA image of the sample using a delay-and-sum back projection reconstruction algorithm.⁶⁸

We note that local temperature rise in the order of few millidegrees can produce photoacoustic signal strong enough for imaging. For instance, one millidegree of temperature rise produces 8 mbar of pressure rise. The ultrasound detectors used for photoacoustic imaging are sensitive enough to record these pressure waves. Moreover, the imaging time is quite short. It has been shown that the photoacoustic cross sectional imaging can be performed as fast as in 3 second imaging time.⁶⁸ If one uses photoacoustic imaging system based on an ultrasound array transducer, one can obtain photoacoustic

Figure. 3. Photoacoustic (PA) signal of BBT-2FT compared with blood. (a) PA signals of blood and BBT-2FT NPs (2 mg/mL) received by 2.25 MHz UST, (b) PA signal as a function of concentration of BBT-2FT NPs. PA signals generated from blood (c) and BBT-2FT NPs (d) in a LDPE tube embedded inside a chicken breast tissue at difference depths, (e) Zoom in version of the PA signal at D4 = 4 cm. (f) The PA signal of blood and BBT-2FT NPs as a function of penetration depth in chicken tissues.

imaging in a single laser pulse. Therefore, the photoacoustic imaging itself will not cause any significant temperature rise to initiate the photothermal therapy process.

Photoacoustic signals from blood/BBT-2FT samples

Figure. 1b shows the UV-VIS-NIR extinction spectrum of the BBT-2FT NPs. It shows high absorption in the NIR wavelength region which indicates the potential of BBT-2FT to act as a PAT contrast agent. To compare the PA signal from animal blood and BBT-2FT NPs, we performed experiments on animal blood sample vs. BBT-2FT NPs inside low-density polyethylene (LDPE) tubes (~ 0.59 mm inner diameter (ID), ~ 0.78 mm outer diameter (OD)). The PA signal received by the UST was band pass filtered (1-10 MHz) and amplified with 50 dB gain. Finally, the signal was digitized by a DAQ card at 25 Ms/s and stored in computer. A total of 7,000 A-lines (1 sec) were collected. Figure. 3a shows the PA signals averaged 700 times from animal blood and BBT-2FT NPs (2 mg/mL). The signal from BBT-2FT NPs is ~ 10 times stronger than that from blood. Figure. 3b shows that the PA signal intensities increased linearly with the concentration of BBT-2FT NPs.

Deep-tissue imaging experiment

To check the feasibility of BBT-2FT NPs as a PAT contrast agent and determine their effective imaging depth at a wavelength of 803 nm, we acquired PA signals of blood and BBT-2FT NPs embedded inside a chicken breast tissue.

The LDPE tube filled with blood or BBT-2FT NPs (2 mg/mL) was embedded in the chicken breast tissue. PA signals were collected when the tube was placed at 1, 2, 3, or 4 cm deep from the laser illuminated tissue surface. The incident laser energy density on the tissue surface area is ~ 0.28 mJ/cm², which is much less than the "maximum permissible exposure

acquired using 2.25 MHz transducer at 1 cm (c), and 2 cm (d) depth from the light illuminated tissue surface.

(MPE)" of 32 mJ/cm² at 803 nm. Figures. 3c and 3d show the PA signals collected from blood and BBT-2FT (2 mg/mL) at different depths (D) by the 2.25 MHz UST. In our current experiments, blood and BBT-2FT NPs were successfully detected in chicken breast tissue at depth of ~ 2.0 cm, and ~ 4.0 cm respectively. The imaging depth can be further increased by using a higher power pulsed laser (for example optical parametric oscillator (OPO) laser pumped by Nd:YAG laser producing more than 70 times stronger energy per pulse).

Deep-tissue imaging experiments were carried out on the sample shown in Figure. 4a, which is made of two LDPE tubes (~ 0.59 mm ID, ~ 0.78 mm OD, ~ 8 mm long), one filled with blood and other filled with BBT-2FT NPs (2 mg/mL). The two LDPE tubes were placed on a chicken breast tissue as shown in Figure. 4a. For imaging they were covered by tissues of various thicknesses as shown in Figure. 4b. The tissue cross-section containing the LDPE tubes was imaged when tissue slices were sequentially placed to make the tubes 1 cm, and 2 cm deep from laser-illuminated tissue surface. Figure. 4c and 4d show the PAT images acquired at 1 cm, and 2 cm depth, respectively. The SNR values of blood, BBT-2FT NPs measured from Figure. 4c are ~ 23 , ~ 35 and that measured from Figure. 4d are ~ 9 , ~ 14 , respectively. Both the tubes were clearly visible at 2 cm under the chicken breast tissues. Our results indicate that BBT-2FT NPs are promising contrast agents for PAT with good PA signal enhancement and image contrast in biological tissues.

Photothermal therapy *in vitro* experiments

We next investigate the *in vitro* photothermal treatment and cytotoxicity with BBT-2FT NPs. HeLa cells (human cervical carcinoma cell lines) cultured in 12-well plates were incubated with 25 $\mu\text{g mL}^{-1}$ BBT-2FT NPs solution for 6 h, after rinsing with PBS twice and being resupplied with fresh DMEM culture medium, then the cells were irradiated with an NIR laser (808 nm and 1.77 W/cm²) for 10 min. Live/dead cells were differentiated by calcein AM (live cells, green fluorescence) and propidium iodide (PI) (dead cells, red fluorescence) co-staining after photothermal therapy treatment (Figure. 5a). Up to 90% cells were killed after treatments of BBT-2FT NPs and laser irradiation. As shown in Figure. 5a, on the boundary of the laser spot, only cells within the laser spot were killed, showing intense homogeneous red fluorescence. The cells outside the region of the laser spot stayed alive, showing strong green fluorescence.

To evaluate the photothermal cytotoxicity of BBT-2FT NPs in a more quantitative way, HeLa cell lines were irradiated under laser over various periods tested by PrestoBlue[®] reagent. Resazurin ($\lambda_{\text{max.abs}} = 600$ nm) in the PrestoBlue[®] reagent, a nonfluorescent blue compound, can be reduced in live cells by metabolism to resorufin ($\lambda_{\text{max.abs}} = 571$ nm), which is red in color and highly fluorescent. Since the number of metabolically active cells proportionally correlates with the reduction level, the absorbance readings can be converted and expressed as

Figure.4. Deep-tissue imaging of blood and BBT-2FT tubes embedded inside chicken breast tissue using PAT system. Photograph of the (a) LDPE tubes with blood and BBT-2FT on chicken tissue, (b) stack of chicken tissue layers inside which the blood and the BBT-2FT tubes were kept. PAT images

the percentage reduction of the PrestoBlue® reagent, indicating the relative cell viability. In the experimental group, cells incubated with BBT-2FT NPs for 6 h in a 96-well plate was exposed to the laser for 5 min. At this time point, there was no apparent change in the viability of the three control groups. However, the experimental group at 5 min showed almost 50% dead cells as compared to the control groups (Figure. 5b). When the irradiation time was prolonged to 10 min, there were nearly no viable cells, resulting in close to 100% cell death in the experimental group (Figure. 5b).

To examine the biocompatibility of BBT-2FT NPs, HeLa cells were incubated with gradient concentration of NPs dispersion for 24 h without laser irradiation. Figure. 5c shows the dose-dependence the cytotoxicity of NPs against HeLa cells. One can see that BBT-2FT NPs show minimal toxicity to HeLa cells without NIR laser irradiation. These results suggest that BBT-2FT NPs can serve as a promising photothermal agent for cancer therapy.

Figure. 5 (a) Fluorescence images of calcein AM/PI co-stained HeLa cells after incubation for 6 h with BBT-2FT NPs ($25 \mu\text{g mL}^{-1}$) after being irradiated by laser (808 nm and 1.77 W/cm^2) for 10 min. (b) Viabilities of HeLa cells after PTT at different laser irradiation time. Cell viability was normalized to the control group without any treatment. Error bars are based on the standard deviations of five parallel samples. (c) Viability of HeLa cells after being incubated with various concentrations of BBT-2FT NPs for 24 h tested by PrestoBlue® reagent without laser irradiation.

Conclusions

We have presented a dual-modal photoacoustic imaging and photothermal therapeutic agent based on BBT-2FT NPs with strong absorption in the NIR region. These NPs exhibit good colloidal stability, obviously stronger photoacoustic signal than blood, higher photothermal conversion efficiency, and excellent photostability. Almost 10 times stronger photoacoustic intensity than blood was detected, and 4 cm tissue penetration depth makes it viable for *in vivo*

applications. Moreover, significant death of HeLa cells was observed due to the hyperthermal effect. These results demonstrate that the BBT-based NPs are promising theranostic agents for cancer imaging and therapy. Further application of these dual functional agents in animals and clinical trial is under investigation, which, if successful, will enable the “see and treat” strategy using a single platform.

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