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Melanin nanoparticles as a novel contrast agent for optoacoustic tomography

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We describe the synthesis and characterization of melanin-like nanoparticles (MNP) as novel contrast agents for optoacoustic tomography. Good dispersion stability of high concentration MNPs in different biological media was achieved with thiol-terminated methoxy-poly(ethylene glycol), which can be used for further functional conjugation. MNP-PEG were found biocompatible with human MCF-7 and 3T3 cells. Cell toxicity of MNPs was found lower than that of gold nanorods for concentrations that provide equal optical absorbance. Optoacoustic tomography images were obtained with Laser Optoacoustic Imaging System (LOIS-3D) from tubes filled with contrast agents and live mice. Imaging of tubes permitted verification of the system resolution <300 μm and sensitivity $\Delta\mu a$=0.03/cm under safe laser fluence of 20 mJ/cm^2. Water suspensions of MNP demonstrated optoacoustic efficiency that is about equal to that of gold nanorods under conditions of equal optical absorption. We conclude that MNPs have the potential for biomedical imaging applications as optoacoustic contrast agents.

1. Introduction

Optoacoustic tomography (OAT) is a biomedical imaging modality that combines the spectral selectivity and the high optical contrast based on variation of optical absorption with the high resolution based on detection of ultrasound generated in tissues with nanosecond laser pulses [1,2]. Typically, lasers emitting in the near-infrared (NIR) spectral range from ~700 nm to ~1300 nm are used for generation of OA signals (and images) due to the relatively weak absorption of biological tissues in this spectral range, also known as the window of optical tissue transparency [3]. Even though OAT resolution is scalable with approximate ratio of depth of imaging to resolution of about 200 [4], the most significant value of this technology is expected from its capability of visualizing deep tissue structures [5–8] and potentially providing high contrast, high resolution quantitative volumetric information about molecular content of biological tissues.

Hemoglobin and oxy-hemoglobin of blood are the main tissue chromophores in the NIR spectral range [9], therefore OAT may be naturally defined as a functional imaging modality for characterization of blood distribution in the live body. On the other hand, not many molecules of biomedical interest possess strong optical absorption in the range of the optical tissue transparency. Therefore, application of contrast agents (CA) that target non-absorbing molecules and cells is important for molecular optoacoustic imaging. A large number of optical and optoacoustic CA has been developed since early 2000s, and many of them found applications in preclinical research using live animal models [10]. The signal amplitude emitted by a contrast agent is proportional to its volume accumulated at the target site. Therefore, nanoparticles, such as gold nanorods, having their volume and the optical absorption coefficient much larger than those of any molecular probe, are thought to be of especially significant value as contrast agents for optoacoustic imaging [11]. All nanoparticle based optoacoustic CA can be divided in two groups: nanoparticles based on exogenous or endogenous chromophores. While CA development in the previous decade was focused on exogenous nanoparticles, recently the deserved attention is gradually shifting towards less toxic nanoparticles based on endogenous molecules [12]. Two of the biggest advantages of using endogenous contrast agents for imaging applications are safety and the possibility of revealing the true physiological conditions, because the physiological parameters are not altered during optoacoustic image acquisition.

1.1. Optoacoustic contrast opportunity offered by Melanin

Melanin molecules have much stronger optical absorption than surrounding skin in the near-infrared region [13]. However, unlike

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hemoglobin and oxy-hemoglobin, imaging of melanin in skin is not used for biomedical diagnostics, with exception of assessment of skin protection from ultraviolet radiation. We would like to employ strong NIR absorption of melanin to enhance optoacoustic contrast of tissues, such as cancerous tumors, which can potentially accumulate significant concentration of melanin nanoparticles. The broad optical absorption spectrum of melanin makes it suitable for optoacoustic imaging with any available laser wavelength [14]. Melanins (may be surprisingly) are widely distributed in many parts of living organisms. Melanins are involved in various functions, including photosensitization, metal ion chelation, thermoregulation, protection from radiation and free radical quenching, a vital property in the regulation of oxidative stress [15,16]. Melanins are usually categorized into two major types according to the difference in precursors and colors: brown-black eumelanins and yellow-red pheomelanins [17]. Unlike fluorescent proteins, melanin cannot be used for studies of subcellular protein distribution and interaction analysis, but it has the advantage of being visible by such noninvasive deep tissue imaging as optoacoustics. An advantage of melanin compared with fluorescent proteins is its very good stability in physiological environment of live animals [18].

Previously, optoacoustic imaging has been used to detect melanin in lymph node metastases from melanoma cancer. Differentiation between blood and melanoma proved to be difficult because both are strong optical absorbers and therefore create comparable optoacoustic signals [19]. OAT was previously proposed for diagnosis, prognosis, and treatment planning of melanotic melanoma (>90% of all melanomas) [20]. Several groups successfully demonstrated gene delivery technique for over-expression of melanin in cells loaded with tyrosinase, which resulted in melanin contrast for optoacoustic (photoacoustic) microscopy [21,22]. Melanin was also shown to be a suitable target for laser-induced thermotherapy [18]. The tyrosinase gene can be utilized as a multifunctional reporter gene for optoacoustic, magnetic resonance and positron emission tomography *in vitro* and *in vivo* [23]. On the other hand, the process of melanin production in transfected cells is quite toxic [24]. For example, epidermal melanocytes are particularly vulnerable to oxidative stress owing to the pro-oxidant state generated during melanin synthesis, and to the intrinsic antioxidant defenses that are compromised in pathologic conditions. Melanin synthesis involves oxidation reactions and superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2) generation, which subject melanocytes to oxidative stress [25]. Therefore, we support promising application of melanin as a contrast agent formulated as nanoparticles. Melanin is an effective scavenger of free radical toxicity. Application of melanin-based nanoparticles has been demonstrated as a protective agent against GNR induced neurotoxicity in mice [26], against ionizing radiation [27] and, thus, MNP may be used as a contrast and protection nanoplatform for different imaging modalities [28]. There are usually two approaches toward fabrication of melanin nanoparticles: nanoparticle formation from melanin isolated from natural sources and synthesis of an artificial MNPs. Natural melanins have been obtained by separation and purification of the pigment from their biological environment and these procedures need to be developed to obtain the unmodified characteristics of natural melanins [29]. Synthetic melanin models are usually prepared by chemical oxidation of dopamine [30] or enzymatic oxidation of precursor molecules such as tyrosine and 3,4-dihydroxy-L-phenylalanine. While physical and chemical properties of melanin are preserved in the process of fabrication, synthetic melanin models usually could not provide the particle shape and were insoluble in water [31]. However, in the past several years there have been developments in synthetic methods to prepare size-controllable melanin-like nanoparticles having a good dispersibility in water and biological media [29,32,33]. High dispersibility and dispersion stability of nanoparticles is critically important for two aspects of *in vivo* applications. The first, administration of the contrast agents has to be made in significantly enhanced concentrations in order to make their optical absorbance competitive with red blood cells. The second aspect is that effective PEGylation of nanoparticles needed for high MNP dispersibility in biological media simultaneously make these nanoparticles invisible to reticulo-endothelial system [34].

Our report is focused on three MNP-related aspects: (i) the dispersion stability of MNP-PEG conjugates, (ii) the toxicity of PEG-MNP conjugates in different cell cultures and *in vivo* and (iii) the investigation of MNP as a contrast agent for optoacoustic imaging.

2. Materials and methods

2.1. Reagents

The chemicals were obtained at the highest purity available and used as received from commercial sources: dopamine hydrochloride (Sigma Aldrich), sodium hydroxide (NaOH, Sigma), hexadecyltrimethylammonium bromide (CTAB, Sigma), ammonia hydroxide (NH_4OH, Sigma-Aldrich), potassium carbonate (K_2CO_3, Sigma-Aldrich), poly (ethylene glycol) methyl either thiol or methoxypolyethylene glycol thiol mPEG thiol, MW 5000, (mPEG-Thiol or PEG, Laysan Bio Inc.), gold(III) chloride trihydrate or chloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$, Aldrich), sodium borohydride ($NaBH_4$, Aldrich), silver nitrate ($AgNO_3$, Sigma- Aldrich). Ultrapure water (18.2 MΩ·cm at 25 °C) was used throughout the work.

2.2. Synthesis of Water Dispersible MNPs.

Water-dispersible MNP were prepared according to the protocol described originally described in [29] by an oxidation and polymerization of 3,4-dihydroxy-phenylalanin (DOPA) with $KMnO_4$ [32]. A total of 50 mg dopamine hydrochloride was dissolved in 20 mL of deionized water. Under vigorous stirring, 40 to 400 μL of 1 N NaOH was added to a dopamine hydrochloride solution at 60 °C. Instead of originally proposed 4 hours [29], we kept the reaction overnight at pH=10 and achieved a more homogeneous distribution of MNPs. The experiments were conducted with 200 μL of sodium hydroxide. The color of the solution turned to pale yellow as soon as NaOH was added and gradually changed from transparent light to very dark brown. After reacting overnight, MNPs were retrieved by dual centrifugation. In contrast to original single centrifugation, we first used low-speed centrifugation (2500 g, 10 min) and collected supernatant discarding pellet of heavy large-sized aggregated materials. Then we performed a high-speed centrifugation (16000 g, 20 min, RT), collected the pellet and washed it twice with deionized water. To increase the working concentration of the MNP solution, high speed centrifugation (16000 g, 20 min) could be repeated.

2.3. Surface Modification of MNP by PEGylation.

For optimization of PEGylation of MNP, using our previous experience with gold nanorods (GNR) [34], we modified the PEGylation method previously reported in [33]. To achieve a better PEGylation, 1.0 mL of 2 mM potassium carbonate (K_2CO_3) was added to 8 mL of aqueous MNP solution (0.5 mg/mL of water), and 1.0 mL of mPEG-Thiol-5000 (molecular weight 5000, Laysan Bio Inc.) was added in concentration 10 mM (i.e. C=5.0 mg/mL). NH_4OH solution (28 wt %) was added to adjust the pH to between 9 and 10 to stabilize the reactive medium [29]. In accord with our previous studies [34], in the final stage of PEGylation we added K_2CO_3 to activate SH group of mPEG-Thiol molecule in order to achieve better binding to the surface of the nanoparticle. After