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Effect of halloysite nanotubes on the structure and function of important multiple blood components

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ABSTRACT

Many researchers have investigated the application of halloysite nanotubes (HNTs) in biomedicine, because of their special nanoscale hollow tubular structure. Although the cytocompatibility of HNTs has been studied, their blood compatibility has not been systematically investigated. In this work, the effect of HNTs on the structure and function of different blood components has been studied, including the morphology and hemolysis of red blood cells (RBCs). Based on scanning electron microscopy (SEM) observations, optical density test and flow cytometry analysis, we found that HNTs can affect the morphology and membrane integrity of RBCs in phosphate buffered saline (PBS) in a content-dependent way. In particular, based on UV–vis absorption spectra, fluorescence spectra and circular dichroism (CD) spectra, HNTs can alter the secondary structure and conformation of human fibrinogen and γ-globulins. In addition, the detection of biomarker molecules C3a and C5a in plasma suggests that HNTs can trigger complement activation. In the blood clotting assay, HNTs were found to significantly prolong the activated partial thromboplastin time (APTT), shorten the prothrombin time (PT) of platelet-poor plasma (PPP), and change the thromboelastography (TEG) parameters of whole blood coagulation. Furthermore, confocal laser scanning microscopy and flow cytometry analysis were used to test intracellular uptake by macrophages, and the cellular uptake of HNTs in the RAW 264.7 was found to be content-dependent, but not time-dependent. These findings provide insight for the potential use of HNTs as biofriendly nanocontainers for biomaterials *in vivo*.

1. Introduction

Because of quantum size effects and their large surface area to volume ratio, nanomaterials have unique properties, compared to their larger counterparts. Besides improving the existing biomedical technologies, they also offer entirely new strategies in healthcare [1,2]. Nevertheless, emerging data has revealed a range of toxic effects from nanomaterials, suggesting that they may lead to unexpected toxicities and unwanted biological interactions [3–5]. While standard methods have been used to evaluate the hazards and risks of conventional or micrometer-sized biomaterials, new and modified methods are needed and must be verified before they can be applied to nanomaterials [6,7]. Nanomaterials may interact with cellular components, disrupt or alter cell function, or create reactive oxygen species [8,9]. Moreover, interactions between nanomaterials and the immune system have been shown to increase immunotoxicity, as reviewed by Dobrovolskaia and McNeil [10].

Since most of the biomedical nanomaterials that are used for treatment and diagnosis usually enter the blood via intravenous injection, the interactions of nanoparticles with blood components (i.e., serum proteins [11], the complement system [12], blood cells [13], and the immune response [10,14], etc.) will determine the clinical application of the nanomaterials.

Halloysite nanotubes (HNTs) are double-layered aluminosilicate clay with a predominantly hollow tubular structure. The empty lumen microstructure and porosity give HNTs a high loading and absorption ability. With a suitable design, they can be viable and inexpensive nanoscale containers for encapsulating biologically active molecules and drugs, as was first demonstrated by Price et al. [15]. As a novel drug delivery system, HNTs have been shown to enable the loading and sustained release of biomacromolecules and drugs [16–18], including proteins [19], genes [20], antibiotics [21], and other drugs [22]. Moreover, HNTs and polymer composites may be useful for medical implants, for example, in bone repair [23], or as hemostatic sponges [24] and tissue engineering scaffold materials [25].

With the emergence of new kinds of nano-capsules, biocompatibility is the main prerequisite for their safe usage in delivering biologically active substances [26]. Current research is exploring the cytotoxicity from cellular exposure to HNTs in concentrations that would be useful for commercial applications [27]. Although the blood compatibility of nanomaterials is critical [28,29], a comprehensive study of HNT blood compatibility has not yet been done.

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In this study, we analyzed the effect of HNTs on the structure and function of blood components, including red blood cell (RBC) hemolysis and morphology, the structure and conformation of key plasma proteins (fibrinogen and γ-globulins), complement activation, and blood coagulation function. Furthermore, we investigated intracellular uptake by macrophages.

2. Materials and methods

2.1. Morphology and size of HNTs

HNT powder (purchased from Sigma-Aldrich) was dispersed in phosphate buffered saline buffer (PBS, pH = 7.4) at a concentration of 0.01 mg/mL. The suspension was bath sonicated for 30 min at a power of 150 W and frequency of 40 kHz. The suspension (200 μL) was dropped onto copper grids and air-dried at room temperature. The morphology of HNTs was observed by transmission electron microscopy (TEM, PHILIPS TECNAI 10, Holland) with an accelerating voltage of 200 kV. The zeta potential of the HNTs (0.01 mg/mL) was determined in electrolyte suspensions (0.001 M KCl, pH = 7.4), using a particle size analyzer (Malvern, Zetasizer Nano ZS90).

2.2. Effect of HNTs on RBCs

HNT powder was directly dispersed in PBS (pH = 7.4) at concentrations of 0.01, 0.1, and 1 mg/mL. A solution of 30% BSA (in PBS) was also used to disperse the HNT powder to investigate its effect on the hemolysis ratio. The suspensions were bath sonicated for 30 min before being used. Blood was obtained from healthy volunteers and treated with sodium citrate (as an anticoagulant). The upper layer of whole blood was carefully removed after centrifugation at 1000 × *g* for 5 min. The remaining RBC pellets were washed three times with PBS, and redispersed with PBS to the initial volume.

2.2.1. Hemolysis ratio

The RBCs suspension (0.5 mL) was incubated with various HNT suspensions (0.5 mL) at 37 °C for 4 h, with the final concentrations of HNTs being 0.005, 0.05, and 0.5 mg/mL. PBS (0.5 mL) or deionized water (0.5 mL) was added to the RBC suspension (0.5 mL) as a negative and positive control, respectively. The supernatant was obtained by centrifuging the suspensions at 1000 × *g* for 5 min. The optical densities (OD) of the supernatants (200 μL) were measured at 540 nm with a plate reader (Multiskan MK3, Thermo Scientific) to detect the amount of released hemoglobin from the RBCs. The hemolysis ratio (%) for the HNTs was calculated using the mean OD for each group as follows: Hemolysis ratio (%) = (ODs − ODn) / (ODp − ODn) × 100%, where ODs, ODn, and ODp correspond to the absorbance values for the experimental, negative control, and positive control groups, respectively. All hemolysis experiments were conducted three times and presented as the mean ± standard deviation.

2.2.2. Morphology of RBCs

RBCs suspensions (0.5 mL) were mixed with HNT suspensions (0.5 mL) in PBS or 30% BSA solution to final concentrations of 0.005, 0.05, and 0.5 mg/mL, and incubated at 37 °C for 4 h. The RBCs were then washed three times with PBS and fixed with 4% paraformaldehyde overnight. The fixed RBCs were deposited on glass slides and dehydrated with an ascending series of ethanol (60, 70, 85, 95, and 100%) for 10 min each. After being air dried and coated with gold, the RBCs were observed with a scanning electron microscope (SEM, Philips XL-30, Holland).

2.2.3. Flow cytometry analysis

Fluorescein isothiocyanate (FITC) was used to label the HNTs by aminopropyltriethoxysilane functionalization, as described previously [30–32]. The binding efficiency of the HNTs to the RBC membrane surface was investigated by flow cytometry. RBC suspensions (1.0 mL) were mixed with the labeled HNT suspensions (1.0 mL) in PBS to final concentrations of 0.005, 0.05, and 0.5 mg/mL, and incubated at 37 °C in the dark for 1 h. The stained RBCs were then centrifuged at 1000 × *g* for 5 min and followed by three washing cycles with PBS to remove the suspended HNTs. The RBCs were fixed by being added to 75% ethanol at 4 °C overnight for the flow cytometry analysis (FACS Aria, Becton, Dickinson and Company, USA).

2.3. Effect of HNTs on the structure and conformation of plasma proteins

Human fibrinogen and human γ-globulins were purchased from Sigma-Aldrich. All proteins were dissolved in PBS to a concentration of 0.16 mg/mL before being used. The protein solutions were mixed with different HNT suspensions (PBS as the control) at an equal volume, and then incubated at 37 °C for 30 min. The final protein concentration was 0.08 mg/mL and the final HNT concentrations were 0.005, 0.05, and 0.5 mg/mL. The solutions were evaluated by UV–vis absorption spectra, fluorescence spectra, and circular dichroism (CD) spectra, as follows:

- UV–vis absorption spectra of the protein solutions, with and without HNTs, were recorded in the 200–400 nm range at room temperature with 1 cm quartz cuvettes on a UV-2550 spectrophotometer (Shimadzu Corporation, Japan).
- Fluorescence spectra with the excitation wavelength of 280 nm were recorded on a fluorescence spectrophotometer (Hitachi F-7000 High-Technologies Corp., Japan). Fluorescence spectra of the proteins, with and without HNTs, were recorded by the fluorescence emission spectra in the 300–450 nm range at room temperature. The slit width for the excitation and emission was set at 5 nm and the scan rate was 1200 nm/min.
- CD spectra were recorded on a CD spectrometer (Applied Photophysics Ltd., Leatherhead, UK) to examine conformational change of the proteins due to the HNTs. All spectra presented the average molar ellipticity of three scans recorded in a nitrogen atmosphere at 25 °C in the 190–260 nm range with a scan rate of 60 nm/min and a curette of 1 cm path length.

2.4. Complement activation by HNTs

To assess complement activation, the concentration for cleavage of complement protein fragments C3a and C5a in the plasma was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Shanghai Shensu Biotech Company, China) according to the manufacturer's instructions. In brief, platelet-poor plasma (PPP), used for the activation studies, was prepared by centrifuging the citrated whole blood at 3000 × *g* for 15 min and collecting the plasma supernatant. The plasma supernatant (200 μL) and HNT suspensions (200 μL) were mixed and then incubated for 2 h at 37 °C, where the final concentrations of HNTs were 0.005, 0.05, and 0.5 mg/mL. The mixtures were then centrifuged at 2000 × *g* for 10 min and the supernatants were collected for the ELISA. All of the complement activation experiments were done in triplicate.

2.5. Effect of HNTs on blood coagulation

Thromboplastin time (APTT) and prothrombin time (PT) were determined with an automatic coagulation analyzer (SF-8000, Beijing Succeeder Company, Beijing, China), according to the manufacture's specifications. PPP was prepared by centrifuging the anticoagulated whole blood at 3000 × *g* for 15 min and collecting the supernatant. PPP (180 μL) and 20 μL of HNT suspension in PBS were incubated for 1 min at 37 °C, where the final concentration of HNTs were 0.005, 0.05, and 0.5 mg/mL. The corresponding APTT and PT assay reagents were added to the mixture and incubated for 1 min at 37 °C, and the