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Inhibition of thrombin by functionalized C_{60} nanoparticles revealed *via in vitro* assays and *in silico* studies

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ABSTRACT

The studies on the human toxicity of nanoparticles (NPs) are far behind the rapid development of engineered functionalized NPs. Fullerene has been widely used as drug carrier skeleton due to its reported low risk. However, different from other kinds of NPs, fullerene-based NPs (C_{60} NPs) have been found to have an anticoagulation effect, although the potential target is still unknown. In the study, both experimental and computational methods were adopted to gain mechanistic insight into the modulation of thrombin activity by nine kinds of C_{60} NPs with diverse surface chemistry properties. *In vitro* enzyme activity assays showed that all tested surface-modified C_{60} NPs exhibited thrombin inhibition ability. Kinetic studies coupled with competitive testing using 3 known inhibitors indicated that six of the C_{60} NPs, of greater hydrophobicity and hydrogen bond (HB) donor acidity or acceptor basicity, acted as competitive inhibitors of thrombin by directly interacting with the active site of thrombin. A simple quantitative nanostructure-activity relationship model relating the surface substituent properties to the inhibition potential was then established for the six competitive inhibitors. Molecular docking analysis revealed that the intermolecular HB interactions were important for the specific binding of C_{60} NPs to the active site canyon, while the additional stability provided by the surface groups through *van der Waals* interaction also play a key role in the thrombin binding affinity of the NPs. Our results suggest that thrombin is a possible target of the surface-functionalized C_{60} NPs relevant to their anticoagulation effect.

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Introduction

With the rapid development of nanotechnology, the application of nanoparticles (NPs) has been greatly expanded in various fields including industry (Bournival and Ata, 2015; Martin et al., 2015), environmental sciences (Zhang et al., 2014), biology (Bhalla et al., 2015; Rabkin et al., 2015), water purification (Abdel-Azzem Hassan et al., 2015; Liu et al., 2015a) and *in vitro* diagnostics and so on. Especially in medicine, NPs are often used to deliver drugs (Keelan et al., 2015; Rittchen et al., 2015; Vaijayanthimala et al., 2015) and imaging agents (Tian et al., 2015; Wu et al., 2015), helping to improve solubility, changing pharmacokinetic profiles and resulting in their entering into systemic circulation. However, the benefits of nanotechnology drug delivery platforms are often tempered by concerns about the safety of these materials. The exposure level to NPs has kept growing in the environment due to industrial pollution (Cao et al., 2012; Liu et al., 2008). When NPs enter into blood, they immediately

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encounter cells, proteins and key components of the coagulation system, such as platelets and plasma coagulation factors, and initiate procoagulant (Ilinskaya et al., 2014) or anticoagulant reactions (Groner et al., 2015; Yang and Song, 2015). Therefore, there are increasing concerns about nanoparticle-induced coagulopathies. The effects of NPs on the blood coagulation system have been reviewed in detail, and an overview of key physicochemical properties of NPs determining their interactions with the components of the coagulation system has been provided (Ilinskaya and Dobrovolskaia, 2013a, 2013b). Different NPs can induce various biological effects due to their distinct features. The manipulation of NP structures helps to regulate their physicochemical properties and even biological effects, so that modified NPs may cause desirable effects (Liu et al., 2017; Wu et al., 2014; Zhou et al., 2011). Therefore, it is of significance to evaluate the interactions between NPs and the major components of the blood coagulation system for us to understand the blood coagulation mechanisms of NPs and thus further assist in designing better drug nano-carriers.

Thrombin, as a typical serine protease enzyme in human blood, plays an important role in both procoagulant and anticoagulant reactions. In the coagulation cascade, thrombin converts fibrinogen into fibrin, which forms part of the blood clot (Bode et al., 1989; Di Cera, 2008). It also activates other blood coagulation factors such as coagulation factors V, VIII, XIII, and protein C (Koeppe et al., 2005). These activities are modulated upon its association with thrombomodulin (Esmon et al., 1986). In addition, thrombin interacts with different cells and induces platelet aggregation (Shuman, 1986). Its enzymatic activity is strongly affected by endogenous protein inhibitors (Di Nisio et al., 2005). The diverse biological functions of α-thrombin rely on its complex tertiary structure comprising different domains that are specialized in specific thrombin interactions. The proteolytically active thrombin molecule consists of a light chain of 36 residues (A-chain) linked by a single disulfide bond to a heavy chain of 259 residues (B-chain) that contains three intrachain disulfide bonds (Matthews et al., 1996). In fact, the crystal structure of human α-thrombin reveals that prominent structural features of the α-thrombin molecule are a catalytic triad His57, Asp102 and Ser195 within a deep canyon-like active site cleft, and two extended surfaces that are mainly composed of positively charged residues known as exosite I and exosite II. The active site canyon contains Leu60, Arg97, Leu99, Ile174, Asp189, Ala190, Cys191, Glu192, Gly193, Ser214, Trp215, Gly216 and Gly219 in addition to the catalytic triad. Exosite I is mainly located on a loop containing residues 70 to 80 and bordered by the residues 37 and the Lys109–110 segment. In its center, the exposed side chains of Tyr76 and Ile82 form a hydrophobic cap, which is surrounded by side chains of lysine 149, 36, 109, 110, 81 and 70, and arginine 77A, 75, 67 and 73, interspersed by a few hydrophobic side chains. The strong positive charge is only partially compensated by the acidic residues Glu77 and Glu80 involved in an ionic cluster beneath the surface. Exosite II contains a small hydrophobic Leu234-based groove, which is surrounded by several basic residues such as Arg93, Arg101, Arg126, Arg165, Arg233, Lys235, Lys236, Lys240 and His91, the positive charges of whose side chain cannot be fully compensated by the adjacent electrostatic field. Thrombin inhibitors can block the activity by binding to any of the three domains: the active site and two exosites. Argatroban is a univalent inhibitor and binds only to the active site (Yu et al., 2016). Exosite I is a substrate recognition site for fibrinogen binding (Teuschl et al., 2017). Anticoagulants that bind directly to exosite I and block its interaction with substrate are called direct thrombin inhibitors, such as recombinant hirudins, bivalirudin, and ximelagatran, either alone or in combination with melagatran. Exosite II serves as the heparin-binding domain (Mehta et al., 2016). Thrombin is inhibited indirectly by low-molecular-weight heparins, because these drugs strongly catalyze the function of antithrombin. Thrombin is an ideal target for pharmacological inhibition in the course of thrombosis treatment.

Few investigations have been reported on interactions between NPs and thrombin. PPACK (Phe[D]-Pro-Arg-Chloromethylketone) modified perfluorocarbon NPs showed thrombin inhibition at sites of acutely forming thrombi, which manifested local clot inhibition even as systemic effects rapidly diminished (Myerson et al., 2011). Gold NPs coated with hybridized self-assembled aptamers exhibited enhanced anticoagulant activity as a result of inhibiting the thrombin-mediated cleavage of fibrinogen (Shiang et al., 2011). Thus, the modified NPs represented a new platform for localized control of acute thrombosis. Engineered and combustion-derived carbon-based NPs have been used to explore their effects on human platelet aggregation *in vitro* and rat vascular thrombosis *in vivo* (Lin et al., 2013; Radomski et al., 2005). The results showed that various carbon NPs, except fullerene(C_{60})-based NPs, stimulated platelet aggregation and accelerated the rate of vascular thrombosis in rat carotid arteries. Various surface-modified fullerenes have been designed to be potential drug carriers (Zhang et al., 2016) since the C_{60} NPs have been reported to exhibit relatively low risk to environmental safety and human health (Coll et al., 2015). Naturally, the potential effects of C_{60} NPs on human physiology have received more attention (Kim et al., 2014; Szwejkowski et al., 2017; Yan et al., 2016). Therefore, we are very curious about the reason why C_{60} NPs did not cause coagulation and eager to know whether or not such anticoagulation behavior of C_{60} NPs has specific molecular targets.

To answer this question, we hypothesized that C_{60} NPs can specifically bind to thrombin *via* certain surface modifications and then inhibit thrombin activity. The objective of this research is to investigate the specific interaction mechanisms between surface-functionalized C_{60} and thrombin, as well as to construct a quantitative nanostructure-activity relationship (QNAR) to predict the thrombin inhibition of surface-modified C_{60} NPs not yet synthesized.

1. Materials and methods

1.1. Materials

Nine fullerene derivative NPs were purchased from Sigma-Aldrich (St. Louis, MO, USA): [6,6]-phenyl C_{61} butyric acid methyl ester (>99.5%), *N*-methylfulleropyrrolidine (99.0%), [6,6]-thienyl C_{61} butyric acid methyl ester (≥99%), fullerene-methyl nipecotate (≥95.0%), 1′,4′-dihydro-naphtho[2′,3′:1,2] [5,6] fullerene C_{60} (97.0%), (1,2-methanofullerene C_{60})-61-carboxylic acid (97.0%), C_{60} pyrrolidine tris-acid (97.0%),

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