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Nanodiamonds as “artificial proteins”: Regulation of a cell signalling system using low nanomolar solutions of inorganic nanocrystals

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ABSTRACT

The blocking of specific protein-protein interactions using nanoparticles is an emerging alternative to small molecule-based therapeutic interventions. However, the nanoparticles designed as “artificial proteins” generally require modification of their surface with (bio)organic molecules and/or polymers to ensure their selectivity and specificity of action. Here, we show that nanosized diamond crystals (nanodiamonds, NDs) without any synthetically installed (bio)organic interface enable the specific and efficient targeting of the family of extracellular signalling molecules known as fibroblast growth factors (FGFs). We found that low nanomolar solutions of detonation NDs with positive ζ-potential strongly associate with multiple FGF ligands present at sub-nanomolar concentrations and effectively neutralize the effects of FGF signalling in cells without interfering with other growth factor systems and serum proteins unrelated to FGFs. We identified an evolutionarily conserved FGF recognition motif, ~17 amino acids long, that contributes to the selectivity of the ND-FGF interaction. In addition, we inserted this motif into a *de novo* constructed chimeric protein, which significantly improved its interaction with NDs. We demonstrated that the interaction of NDs, as purely inorganic nanoparticles, with proteins can mitigate pathological FGF signalling and promote the restoration of cartilage growth in a mouse limb explant model. Based on our observations, we foresee that NDs may potentially be applied as nanotherapeutics to neutralize disease-related activities of FGFs *in vivo*.

1. Introduction

Since the upswing of nanomedicine in the early 1990s, the use of nanoparticles has strongly influenced the quality of treatment of various diseases and pathogens. The low toxicity of some nanoparticles, their high surface to volume ratio and the possibility of polyvalent binding sites on their surface have enabled the

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application of nanoparticles in targeted drug delivery systems, of theranostic nanoparticles that perform simultaneous therapeutic intervention and monitoring of the treatment, and of stimulus-responsive systems based on hybrid nanoparticles with a broad structural range [1,2]. Overcoming some of the current barriers in cancer treatment, such as poor circulation times, drug resistance and off-target toxicity, using nanotechnology-based systems catalysed the development of a number of drug delivery nanosystems that have already passed the translation phases and are currently marketed as drugs [3]. More recently, promising new directions utilizing nanoparticles for the selective binding or sequestration of biologically active and regulatory compounds have also been investigated. Such nanoparticles called "artificial proteins" [4] take advantage of the remarkable similarity of some nanoparticles to biomolecules, particularly to globular proteins. Artificial proteins are however created to mimic chemically the protein surfaces and typically do not contain proteins. The analogy between proteins and artificial proteins involves not only their comparable overall dimensions and surface charge but in general the analogous steric, thermodynamic and kinetic behaviour of nanoscale structures in an aqueous environment [5].

Similar to biomolecules, nanoparticles can thus bind, mimic, or block specific proteins in living organisms [6]. The use of nanoparticles therefore offers attractive alternative opportunities to small molecule-based therapeutic interventions. For example, the selective binding of one component and consequent blocking of a specific protein-protein recognition using non-toxic nanoparticles can have a strong impact on the control of key disease-related processes. However, owing to issues such as the lack of well-defined binding pockets, developing nanoparticles that selectively modulate protein-protein interactions remains highly challenging.

In the past decade, advanced experiments exploring the potential of artificial proteins have demonstrated that a molecularly designed nanoparticle interface can inhibit or regulate protein-protein interaction in structurally diverse systems. The seminal works in this field include the selective inhibition of cytochrome *c* peroxidase and cytochrome *c* interaction using gold nanoparticles of appropriate size coated with simple ligands terminated only with carboxyl groups [7,8] and the selective disaggregation of prion aggregates using dendrimeric nanoparticles, leading to the elimination of prion molecules from neuroblastoma cells [9]. The high surface-to-volume ratio of nanoparticles has been later advantageously used also for sequestration of target molecules using long affinity DNA polymers attached to the nanoparticle surface [10], and for the capture of a broad range of pathogens and toxins related to sepsis from the blood using opsonin-modified magnetic nanobeads [11,12]. Attachment of peptide fragments to gold nanoparticles was used for creation of artificial antibodies [13] and very recently, an *in vivo* study on mice revealed the high potential of nanoparticles for preventing receptor phosphorylation and downstream endothelial growth factor-dependent cell migration and invasion into the extracellular matrix [14]. These and other examples [15] constitute strong evidence that the regulation of protein-protein interactions using nanoparticles can be used to control key biological processes.

The nanoparticles used for the selective control of protein-protein interactions have always been decorated with a bio-nanointerface consisting of (bio)organic molecules and/or polymers. The organic functional groups presented on nanoparticles and their flexible arrangement, however, can cause unwanted interactions with immune cells [16], which are observed even for the commonly used PEG coatings [17]. Here, we address a key task: is it possible to achieve direct control of the crystalline nanoparticle surface (without further organic modification) to obtain highly selective protein binding that can effectively compete with natural protein-protein interactions and mitigate their biological effects? In practice, this task requires identifying pairs of proteins and inorganic interfaces that show much stronger mutual interactions than the formation of the protein corona, which occurs immediately upon the exposure of a nanoparticle to a biological environment [18].

In our screening, we identified such a specifically interacting pair. We show here that one type of nanosized diamond crystals (nanodiamonds, NDs) [19] without any synthetically installed organic or bioorganic interface already display, at low nanomolar concentration, extremely strong interactions with members of the fibroblast growth factor (FGF) family of growth factors and morphogens. The FGF system represents a major molecular system by which cells sense their extracellular environment and respond to communication signals during development, life and disease. Four human FGF-receptors exist (FGFR1-4) which respond to communication signals delivered by at least 18 FGF ligands. The importance of FGFR signalling is further emphasized by evidence of their role in disease. Many pathological conditions arise from aberrant FGF/FGFR signalling, including several types of cancer, developmental defects and metabolic disorders [20,21].

We show that NDs selectively neutralize FGF signalling (Fig. 1A) without interference with serum proteins and other tested FGF-unrelated growth factor systems, including EGF (epidermal growth factor), NGF (nerve growth factor), TGFβ1 (transforming growth factor β1), IL6 (interleukin 6) and IFNγ (γ-interferon). We identify the evolutionarily conserved FGF region responsible for the selectivity of interaction with ND and discover the molecular principles responsible for the unprecedented strength of the FGF-ND interaction. Finally, we demonstrate that these purely protein-inorganic nanoparticle interactions are strong enough to mitigate pathological FGF signalling and promote the restoration of cartilage growth in a mouse limb explant model.

2. Results and discussion

2.1. NDs with positive ζ-potential inhibit FGF signalling in cells

To test the nanoparticle-based inhibition of FGF signalling in cells, we used NDs, which form a diverse group of carbon nanomaterials based on the diamond core [22,23]. NDs can be classified into three basic types: detonation, high-pressure high-temperature (HPHT), and chemical vapour deposition NDs. The detonation NDs mostly used in this study are very small (<5 nm) nanoparticles that can easily be excreted from the body by glomerular filtration [24]. They are tolerated long term and are non-toxic [25] even if applied at multiple clinically relevant doses as drug delivery nanosystems in rats and primates [26]. Because of the presence of both sp^3 and sp^2 hybridized carbon atoms at the surface [27], detonation NDs provide a wide range of anisotropic surface atomic arrangements consisting only of C, O and H atoms [28,29]. The resulting surface charges at facets [30] and uniquely organized interfacial water layers [31,32] can lead to interfaces with opposite ζ-potentials ranging approximately from −50 to +50 mV [33–36]. This disparity between ζ-potentials results in different affinities of particular proteins to NDs and in their diverse adsorption dynamics, attachments and conformations on the ND surface [37,38].

Based on these assumptions, we first focused on distinguishing the dominant charge effects on the ND surface that contribute to the inhibition of FGF signalling in cells. We selected three representative types of ND: detonation NDs with either positive or negative ζ-potentials (**ND1** and **ND-PL**, respectively) and oxidized **ND-HPHT** with negative ζ-potential (Fig. 1B). The particles showed features characteristic for the particular ND types: elemental composition and infrared spectra are presented in Table S1 and