Nano Today (2013) 8, 11—20

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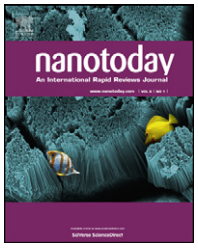

RAPID COMMUNICATION

Degradable polymeric nanocapsule for efficient intracellular delivery of a high molecular weight tumor-selective protein complex

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Received 29 May 2012; received in revised form 4 November 2012; accepted 26 December 2012
Available online 1 February 2013

KEYWORDS
Nanogel;
Core—shell;
Redox-responsive;
Apoptosis;
Breast cancer

Summary The development of stimuli-responsive, nano-scale therapeutics that selectively target and attack tumors is a major research focus in cancer nanotechnology. A potent therapeutic option is to directly arming the cancer cells with apoptotic-inducing proteins that are not affected by tumoral anti-apoptotic maneuvers. The avian virus-derived apoptin forms a high-molecular weight protein complex that selectively accumulates in the nucleus of cancer cell to induce apoptotic cell death. To achieve the efficient intracellular delivery of this tumor-selective protein in functional form, we synthesized degradable, sub-100 nm, core—shell protein nanocapsules containing the 2.4 MDa apoptin complexes. Recombinant apoptin is reversibly encapsulated in a positively charged, water soluble polymer shell and is released in native form in response to reducing conditions such as the cytoplasm. As characterized by confocal microscopy, the nanocapsules are efficiently internalized by mammalian cells lines, with accumulation of rhodamine-labeled apoptin in the nuclei of cancer cells only. Intracellularly released apoptin induced tumor-specific apoptosis in several cancer cell lines and inhibited tumor growth *in vivo*, demonstrating the potential of this polymer—protein combination as an anticancer therapeutic.

Abbreviations: NC, nanocapsule; APO, apoptin; S—S, disulfide bonded; AAm, acrylamide; APMAAm, *N*-(3-aminopropyl)methacrylamide; MBP, maltose binding protein; Rho, rhodamine; ND, nondegradable; HFF, human foreskin fibroblast.

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http://dx.doi.org/10.1016/j.nantod.2012.12.003

Introduction

The most desirable anticancer therapy is both potent and specific toward tumor cells [1,2]. Many conventional small molecule chemotherapeutics do not discriminate between cancerous and normal cells, cause damage to healthy tissues, and are therefore unable to be administered at high dosage. In contrast, cytoplasmic and nuclear proteins that selectively alter the signaling pathways in tumor cells, reactivate apoptosis and restore tissue homeostasis, can delay tumor progression with less collateral damage to other tissues [3—6]. Using stimuli-responsive nanocarriers for the intracellular delivery of such proteins, including human tumor suppressors [7] and exogenous tumor-killing proteins [8—10]), is attractive as a new anti-cancer therapy modality.

Apoptin is a 121-residue protein derived from chicken anemia virus [9]. When transgenically expressed, apoptin can induce p53-independent apoptosis in a variety of tumor and transformed cells [11,12], while leaving normal and untransformed cells unaffected [13]. Apoptin exists as a globular multimeric complex, composed of thirty to forty subunits, with no well-defined secondary structure [14]. While the exact mechanism of the tumor selectivity is unresolved, apoptin is known to translocate to the nucleus where tumor-specific phosphorylation at residue Thr108 takes place, leading to accumulation of apoptin in nucleus and activation of the apoptotic cascade in tumor cell [15]. In normal cells, apoptin is not phosphorylated at Thr108 and is located mostly in the cytoplasm, where it aggregates and undergoes degradation [16]. Because of the high potency in inducing this exquisite tumor-selective apoptosis, apoptin has been investigated widely as an anti-tumor therapeutic option [9]. Different gene therapy approaches have been used to administer apoptin to mouse xenograft tumor models, in which significant reduction in tumor sizes and prolonged lifespan of mice have been observed without compromising the overall health [17—19]. However, as with other gain-of-function therapy candidates, *in vivo* gene delivery approaches using viral vectors may lead to unwanted genetic modifications and elicit safety concerns [20]. While protein transduction domain (PTD)-fused apoptin has been delivered to cells [21,22], this approach suffers from inefficient release of the cargo from endosomes and instability of the unprotected protein [23]. Development of nanoparticle carriers to aid the functional delivery of apoptin to tumor cells is therefore desirable [24].

We chose to work with recombinant maltose-binding-protein fused apoptin (MBP—APO) that can be solubly expressed from *Escherichia coli*, whereas native apoptin forms inclusion bodies [14]. MBP—APO has been shown to similarly assemble into a multimeric protein complex, which exhibits the essential functions and selectivity of native apoptin [14]. Nanoparticle-mediated delivery of functional MBP—APO poses unique challenges [25]. First, MBP—APO preassembles into large complex with an average diameter of ~40 nm and molecular weight of ~2.4 MDa [14]. To achieve nanocarrier sizes that are optimal for *in vivo* administration (~100 nm) [26], a loading strategy that forms compact particles is desirable. Second, in order to maintain the multimeric state of functional MBP—APO, the protein loading and releasing steps need to take place under very mild, physiological conditions in the absence of surfactants. Lastly, the nanocarrier must completely disassemble inside the cell to release the MBP—APO in its native and unobstructed form to ensure the correct spatial presentation of key residues within the apoptin portion, including the nuclear localization/export signals, the phosphorylation site and other elements important for downstream signaling.

In the current study, we selected a polymeric nanocapsule (NC) strategy for the functional delivery of MBP—APO, in which the protein complex is noncovalently protected in a water soluble polymer shell (Fig. 1). This slightly positively charged shell shields the MBP—APO from serum proteases and surrounding environment, while enabling cellular uptake of the polymer—protein complex through endocytosis [27]. The polymeric layer is weaved together by redox-responsive cross-linkers containing disulfide bond (S—S) that can be degraded once the NCs are exposed to the reducing environment in cytoplasm [28]. No covalent bonds are formed between the protein cargo and the polymer shell, which ensures complete disassembly of the capsule layer and release of native MBP—APO inside the cell. Using this approach, we show that MBP—APO can be efficiently delivered to induce apoptosis in cancer cell lines selectively both *in vitro* and *in vivo*.

Materials and methods

Materials

N-(3-aminopropyl)methacrylamide hydrochloride was purchased from Polymer Science, Inc. CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) reagent was purchased from Promega Corporation. APO-BrdU™ TUNEL Assay Kit was purchased from Invitrogen. *In Situ* Cell Death Detection Kit, POD; was purchased from Roche Applied Science. Female athymic nude (*nu*/*nu*) mice, 6 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). All other chemicals were purchased from Sigma—Aldrich and used as received.

Protein nanocapsule preparation

The concentration of protein was diluted to 1 mg/mL with 5 mM sodium bicarbonate buffer at pH 9. Then 200 mg/mL acrylamide (AAm) monomer was added to 1 mL of protein solution with stirring at 4 °C. After 10 min, the second monomer, *N*-(3-aminopropyl)methacrylamide (APMAAm), was added while stirring. Different cross-linkers, *N*,*N*′-methylene bisacrylamide for ND NC and *N*,*N*′-bis(acryloyl)cystamine for S—S NC, were added 5 min after the addition of APMAAm. The polymerization reaction was immediately initiated by adding 30 μL of ammonium persulfate (100 mg/mL, dissolved in deoxygenated and deionized water) and 3 μL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. The polymerization reaction was allowed to proceed for 60 min. The molar ratios of AAm/APMAAm/cross-linker used were