Size dependent PASylation critically regulates H-Ferritin nanocage stability and functionality

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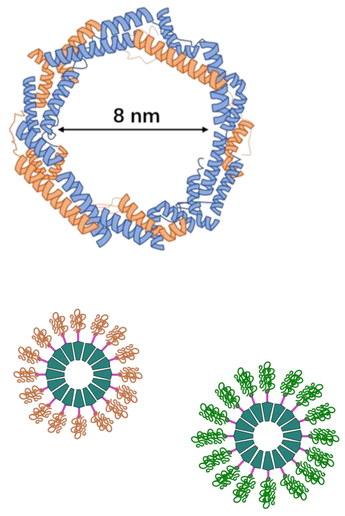
Introduction

Over the past two decades, cancer nanomedicine has focused on nanosystems able to **specifically deliver anticancer drugs to tumors**, improving efficacy while reducing side effects. Among these, **H-ferritin nanocages (HFn)** stand out due to their **biocompatibility, low immunogenicity, and natural tumor homing** via the transferrin receptor (TfR1), commonly overexpressed in cancers [1].

HFn are characterized by a **hollow cavity** (8 nm) and can **efficiently encapsulate drugs and imaging agents** (e.g., doxorubicin and indocyanine green), showing strong results in vitro and in animal models [2-7].



However, their clinical performance is limited by **short circulation time and rapid clearance**, leading to **suboptimal tumor accumulation.**

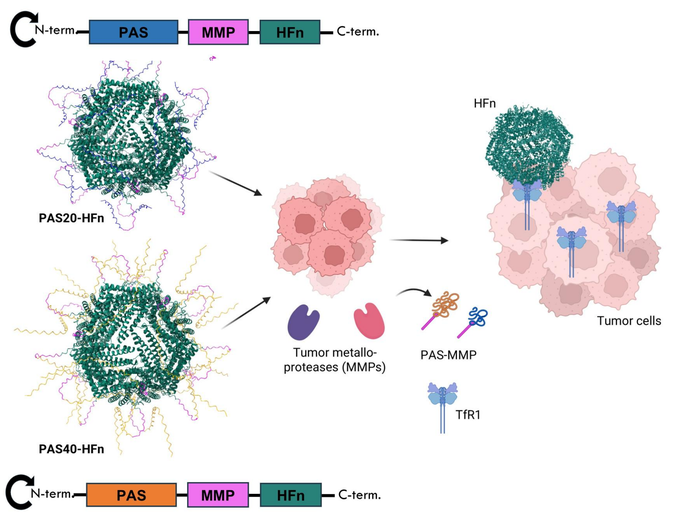
To overcome these limitations, surface engineering strategies have been explored [8]. Among them, **PASylation**, which is the addition of a flexible sequence of proline (P), alanine (A), and serine (S), **resulted in improved stability, circulation time, and bioavailability while preserving tumor targeting** [9]. PAS domains ≥ 40 amino acids have been explored, displaying improved in vivo performance [10].

At present, the effects of shorter PAS sequences remain unknown.

Aim

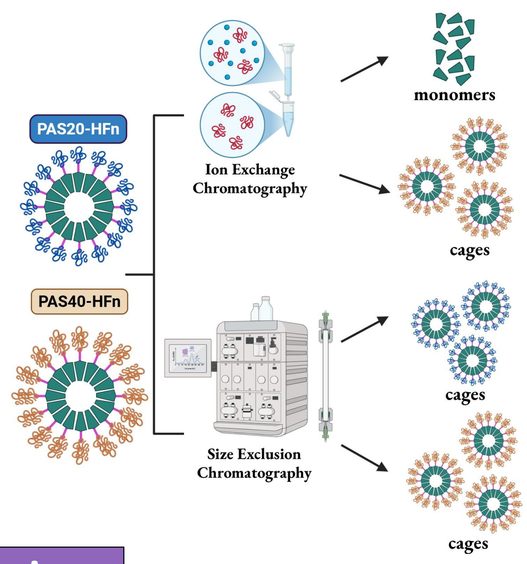
Investigate how shorter PAS chains influence HFn-nanocage stability and functionality.

Nanocages design



PAS sequences of different lengths (40 and 20 amino acids) were inserted in N-term. position followed by a sequence cleavable by Tumor metalloproteases (MMP).

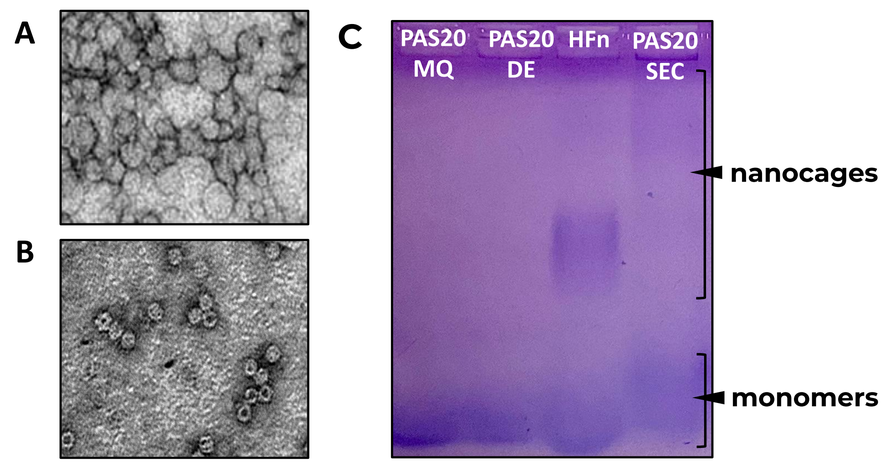
Different behavior of PAS20-HFn and PAS40-HFn



PAS20-HFn
- instability of its quaternary structure
- fail to form nanocages in charge–rich environment

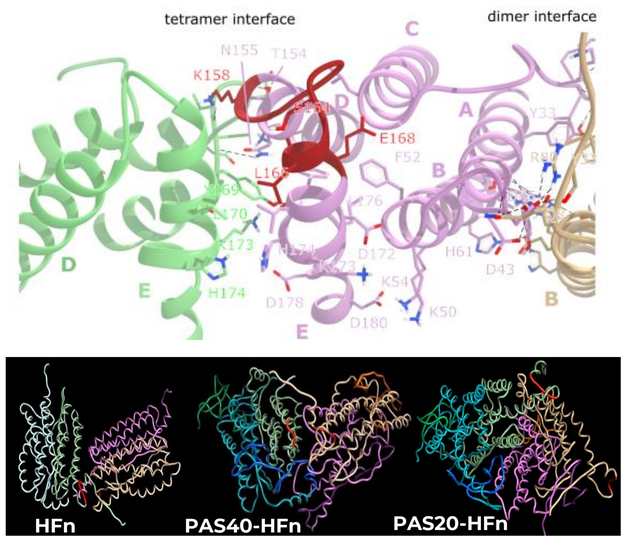
PAS40-HFn
Stable and monodisperse nanocage

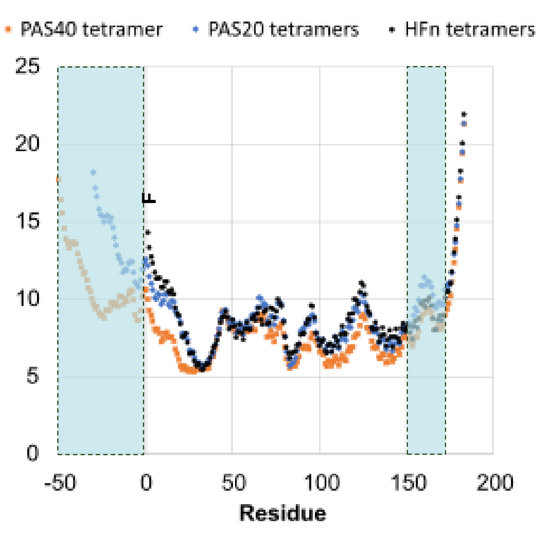
PAS20-HFn characterization



Absence of PAS20-HFn nanocages when purified with IEC (A), while SEC allows PAS20-HFn nanocages purification (B). The charged resin (MonoQ Sepharose) used for IEC purification disrupts nanocages (C).

Molecular dynamics





Molecular dynamics revealed high flexibility of **PAS20-HFn**, which displaces the **158–168 regions** located on adjacent dimers in the tetramer, involved in stabilizing quaternary structure interactions.

PAS20-HFn functional alterations

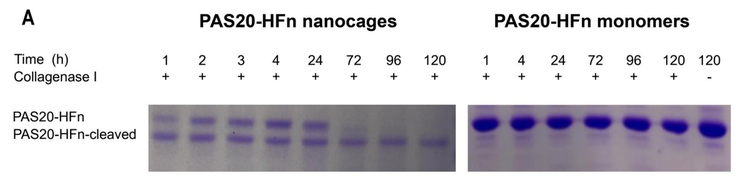
ICG loading

	PAS40-HFn@ICG	PAS20-HFn@ICG	HFn@ICG
ICG encapsulated (mg)	1.50 ± 0.67	0.22 ± 0.06	1.29 ± 0.31
% recovery	26.66 ± 7.52	1.08 ± 0.31	31.07 ± 7.87

pH-dependent method

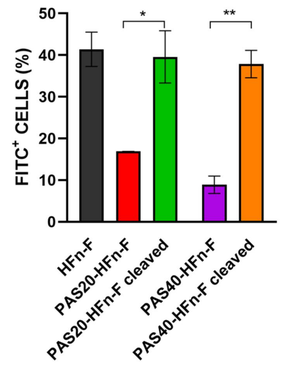
Cleavage assay

Complete PAS domain removal observed in nanocages after 120h; no cleavage in monomers fraction.



Binding assay

Following PAS domain removal, binding to the TfR1 receptor is restored in both mutants.



Conclusions

- ✓ PASylation exert a bipolar and size-dependent effect of HFn nanocages
- ✓ While longer PAS domain enhance stability and functionality, excessively short PAS sequences destabilize oligomerization and compromise drug loading
- ✓ PAS length is a critical design parameter for engineering effective HFn- based nanomedicines

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