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Apoferritin and Dps as drug delivery vehicles: Some selected examples in oncology

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ABSTRACT

Background: The ideal nanoparticle should be able to encapsulate either pharmaceutical agents or imaging probes so that it could treat or image clinical tumours by targeting the cancer site efficiently. Further, it would be an added advantage if it demonstrates: small size, built in targeting, biocompatibility and biodegradability. Ferritin, which is an endogenous self-assembling protein, stores iron and plays a role in iron homeostasis. When iron atoms are removed apoferritin (AFt) is formed which consists of a hollow shell where it can be used to load guest molecules. Due to its unique architecture, AFt has been investigated as a versatile carrier for tumour theranostic applications. DNA-binding protein from starved cells (Dps), which also belongs to the ferritin family, is a protein found only in prokaryotes. It is used to store iron and protect chromosomes from oxidative damage; because of its architecture, Dps could also be used as a delivery vehicle.

Conclusions: Both these nano particles are promising in the field of oncology, especially due to their stability, solubility and biocompatibility features. Further their exterior surface can be modified for better tumour-targeting ability. More studies, are warranted to determine the immunogenicity, biodistribution, and clearance from the body.

General perspective: This review discusses a few selected examples of the remarkable *in vitro* and *in vivo* studies that have been carried out in the recent past with the use of AFt and Dps in targeting and delivery of various pharmaceutical agents, natural products and imaging probes in the field of oncology.

1. Introduction

In recent years nanotechnology applications have increased widely and have shown immense potential to bring benefits in many areas of medicine [1]. Nanomaterials have sizes ranging from 1 to 100 nm, and they could be natural, incidental or manufactured [2]. Hence, a new field of nanomedicine was established at the interface of nanotechnology and medicine, that could bring substantial advances in the prevention, diagnosis and treatment of diseases [3]. Currently, nanomedical research focuses on oncology, where much attention is directed on targeting the disease by efficient delivery. Drug delivery is a technique that administers drugs to accomplish the therapeutic impact in humans and animals and has been at the forefront of nanomedicine [4]. Thus, precision delivery of the drug will enhance therapeutic efficacy and reduce the burden of toxicity due to non-specific interaction with healthy cells, and the amount of drug accumulation in healthy cells, which may minimise toxicity [1,5].

Cancer is a complex disease and a leading cause of death in economically developed countries; it is also the second cause of death in developing countries. Moreover, the number of cases diagnosed in the future is projected to increase [6,7]. Cytotoxic chemotherapies have widely been in use with promising outcomes. However, chemotherapy treatment is often limited by the toxic side effects caused to healthy cells [8]. Additional challenges are associated with chemotherapy drugs

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including multidrug resistance, rapid drug clearance and limited targeting. Nanomedicine has the potential to offer solutions to overcome some of these limitations associated with chemotherapeutic drugs [9].

Nanoscale drug delivery vehicles with nanoscale dimensions and high surface-to-volume ratios have attracted considerable attention for delivery of both synthetic and natural agents and for applications including cancer treatments, tissue engineering and agriculture [10,11]. Numerous potential benefits include controlled drug release, targeting tumour rather than healthy cells, altering the pharmacokinetics and biodistribution of the drug, overcoming biological barriers in the body and increased accumulation of drug at the tumour site due to the enhanced permeability and retention (EPR) effect [12]. These properties make nanoparticles promising tools for the diagnosis, imaging and treatment of cancers [13].

Nanoparticles can be delivered via several routes of administration, such as oral, vascular, and inhalation. To date, the different nanoparticles that have been considered for drug delivery comprise mesoporous silica, metal nanoparticles, lipids, polymers, nanogels and proteins [10,14,15]. These systems have been used to deliver therapeutics efficiently by targeting the specific diseased site [16]. However, it should be noted that biological barriers in humans and animals represent a challenge to drug delivery systems. Engineering nanoscale delivery systems could enable not only targeted delivery [17,18], but also stimulate responsive drug release, that is demonstrated for cancer agents [19]. Identifying and understanding the mechanisms of delivery and uptake of the therapeutic agents delivered using nanoscale vehicles is essential to circumvent environmental and biological constraints [4]. Among various nanoscale materials explored to date, protein capsules offer significant benefits due to their biocompatibility, uniform size, opportunities for functionalization [20] and engineering [21] to enable targeted and specific uptake [22].

Here we focus on protein drug delivery using the iron-free form of ferritin, apoferritin (AFt) and DNA-binding protein from starved cells (Dps) as versatile nano carriers for anticancer drug delivery and theranostic applications [15,23]. Proteins are among the most versatile building blocks in nature programmed for a specific function. AFt and Dps possess a protein cage like architecture, comprising of a small number of subunits self-assembled into symmetrical structures. Thus, the interior and exterior surfaces of the cage-like structure can be exploited for potential drug delivery and targeting. Mammalian AFt is widely researched as a nano carrier for applications in the clinic [24]. In this review we discuss selected examples in development of strategies for protein encapsulation of anticancer agents, and advances made in understanding their activity compared to unencapsulated agents. We also compare delivery of anticancer agents using horse spleen AFt to synthetic human heavy chain AFt, as well as the smaller protein capsule Dps.

2. Proteins as nanomaterials

The use of protein-based nanoparticles for drug delivery has grown rapidly [25]. Proteins are versatile biopolymers formed by the condensation of amino acids in a linear chain. Each protein performs specific functions, for instance, transportation, catalysing metabolic functions, assisting growth and maintenance. Proteins naturally exist at various sizes ranging from large to the nanoscale. Protein nanotechnology is an evolving field and a variety of proteins have been used as nanoparticles. Albumin-bound nano deliveries are currently in clinical use. For example- nab-paclitaxel (Abraxane),-protein-encapsulated paclitaxel, is used in patients with metastatic breast cancer, metastatic non-small cell lung cancer and metastatic pancreatic cancer [26–28].

One of the first protein nanostructures to be evaluated was the cage-type proteins where these proteins possess an internal hollow core [29]. This kind of protein architecture is suitable for drug delivery, as it has an interior cavity with a packed protein shell, and has a homogeneous size distribution. Current investigations have shown the potential of protein cages for delivering numerous therapeutics and imaging agents. As such, the hollow core of the protein allows materials to be encapsulated within the enclosed shell [30]. Therefore, the release of the drug can be regulated by the pore size of the protein cage and the drug concentration difference between the interior and exterior of the protein cage. Also, various stimuli such as temperature, pH, and light may trigger this process. Furthermore, protein cages are usually stable in physiological environments, offering protection to the encapsulated therapies [31].

Protein cages can be effectively modified to target diseased tissue as the surface area and the interior cavity can be genetically and chemically modified without affecting the entire architecture of the protein, thus, incorporating features, such as precise targeting and imaging [31,32]. Moreover, protein-based nanoparticles are biocompatible, biodegradable where they are readily degraded into biocompatible and non-toxic amino acids, and they are easy to manipulate [31]. Interestingly, protein nanoparticles can also cross the blood-brain barrier (BBB), a process denied to most conventional drugs delivered intravenously [30]. The BBB is a semipermeable barrier that controls the movement of molecules, ions and cells crossing into the extracellular fluid of the central nervous system. Thus, this barrier helps to protect the central nervous system from oxidants, pathogens, toxins, and heavy metals [33].

On the other hand, treatment of brain tumours with cancer chemotherapy drugs and targeted therapy is limited by the BBB. Nevertheless, technological advances in protein nanoparticles have demonstrated great promise in treating brain cancers [34]. Therefore, protein nanoparticles can be used in various targeted cancer therapies [30]. Protein nanoparticles are commonly derived from viruses, heat shock proteins, Dps, and ferritins. As such, proteins have been investigated as versatile drug delivery systems. However, only a very limited number of protein nanoparticles have reached the clinic such as Abraxane [24].

3. Apoferritin as a delivery carrier

Apoferritin (AFt; iron-free ferritin) is a protein capsule widely considered for drug delivery. Ferritin is one of the most studied proteins after haemoglobin and was first discovered by Laufberger in 1937, who purified ferritin by crystallisation with cadmium salt [35]. This ubiquitous protein is found in a diverse group of living organisms [36] and includes subfamilies of canonical ferritin, bacterioferritin found only in bacteria and archaea, and the smaller Dps. One of the major differences between canonical ferritin, and bacterioferritin is the presence of twelve heam groups located at the subunit interfaces. However, they both share the same quaternary structure. Lately, archaeal ferritin such as ferritin from *Archaeoglobus fulgidus* has been investigated by many researchers as they have been versatile vehicles in biological applications due to their thermostability, and their ability to assemble/disassemble depending on various ionic concentrations. For instance, ferritin from *A. fulgidus* is able to fully assemble at 0.5 M NaCl. Further, this ferritin assembles in a unique manner into a roughly spherical shell with tetrahedral assembly, which results in four large pores with a diameter of ~45 Å. This provides easy movement of larger molecules in and out of the cavity. However, this type lack selectivity towards eukaryotic cells as they do not possess intrinsic tumour targeting features compared to canonical ferritins, which might be an issue when targeting tumours, thus more investigations are warranted in this regard [26,37]. Dps are dodecameric proteins, and their details are discussed under Section 5.

Ferritin is used to store iron, and prevents the accumulation of toxic levels in living organisms [38,39]. The role of iron storage has been widely studied in canonical ferritins found in both prokaryotes and eukaryotes such as *Escherichia coli* and higher orders of eukaryotes such as horses and humans. In contrast, there are fewer studies on bacterioferritins and Dps. However, there can be considerable differences among the physical and chemical characteristics of all these three types [37]. In mammals, ferritins exist intracellularly in the cytosol, nucleus and mitochondria where iron atoms are mainly stored. Extracellular

ferritins are found in blood plasma as well in synovial and cerebrospinal fluids. When iron atoms are removed from ferritin, AFt is formed (Fig. 1a-b). The protein cage of AFt is composed of 24 subunits, which self-assemble into a hollow cage consisting of an outer diameter of 12 nm, and an inner diameter of 8 nm [40]. An individual subunit consists of a long 4-α-helix bundle with an additional short α-helix lying at an angle of ~60° to the bundle axis at the C-terminal side of the amino acid chain. The interior of AFt can accommodate up to a maximum of 4500 iron atoms. AFt has 14 channels formed at the subunit intersections with diameters of 3–4 Å for exchange of cargo [41]. Eight of those channels are hydrophili, while the remaining six channels are hydrophobic, allowing export of both hydrophilic and hydrophobic drugs and/or imaging agents when encapsulated [42,43].

The AFt nanocage is pH-responsive, and it disassembles at low and high pH (pH ≤ 2.0 or pH ≥ 11.0) and (re)-assembles at neutral pH (7.0) facilitating the encapsulation of drugs and imaging probes. After the encapsulation process, the resulting mixture is dialysed to remove any unencapsulated agent, centrifuged, and the supernatant is stored at a low temperature (4 °C) (Fig. 1c) [23,45]. Although, this approach is more frequently used, there are other methods such as the use of urea or guanidine hydrochloride (GuHCl) to dissociate and to reassemble the protein at different concentration gradients [23,46–50]. Also, a simple method such as utilising diffusion has been used where AFt is incubated with small molecular weight drug molecules for a certain period, allowing the drug to pass through the channels of the AFt cage at a low temperature setting such as 4 °C. The resulting solution is dialysed against buffer solutions to remove any unencapsulated material for >12 h. Subsequently, the solution is centrifuged at high speed to remove any impurities and the supernatant is stored at a low temperature such as 4 °C. This method is also referred to as the nano reactor route (Fig. 1c) [51]. Hence, the self-assembly and channel sensitivity features of AFt have been very useful in preparation of nanoparticles with various agents encapsulated. The number of guest molecules that are trapped within the cavity may vary between ~10 to ≥500 molecules. At the same time, the encapsulation efficiency rate may depend on the guest molecule which has been loaded into the hollow core. This rate may range between ~10% and 85% [23,52]. Many researchers have confirmed by various methods such as sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), size-exclusion chromatography (SEC), dynamic light scattering (DLS), transmission electron microscopy (TEM) (Fig. 2), that entrapment of drug molecules

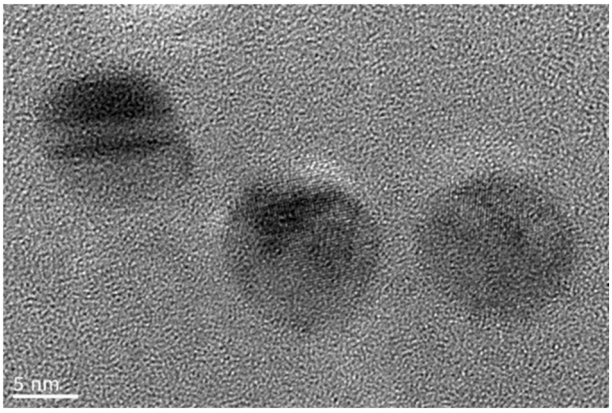

Fig. 2. TEM image of gefitinib-AFt showing nanoparticles with diameter of ~13 ± 0.5 nm [53].

does not disrupt the structure of AFt, and endorse that the AFt nanoparticles possess and retain their spherical shape after encapsulation. Further, zeta potential and time-of-flight secondary ion mass spectrometry (ToF-SIMS) measurements can be used to confirm the absence of drug attachment to the AFt exterior upon agent loading [48,53,54].

The mammalian AFt protein subunits are of two types; heavy and light chains with a molecular weight of 21,000 Da and 19,000 Da, respectively. The two classes of subunits share nearly identical homology. Nevertheless, heavy and light subunits have distinct functions according to their structures; for instance, the heavy chain contains a ferroxidase centre and can convert free toxic ferrous to ferric ions, which is also known as iron oxidation, that prevents damage of cells from the Fenton reaction. The heavy chain subunits also facilitate the accumulation of iron, while the light chain subunits lack ferroxidase activity but facilitate iron nucleation and mineralisation [55,56]. Further, the heavy and light chain subunits are expressed in different ratios, depending on the tissue type examined. For example, the liver and spleen have a high content of light chain subunits [57]. In contrast, tissues such as the brain and heart have a predominance of the heavy chain subunits. This is because they require protection from the potentially toxic effects of free iron which provokes the generation of hydroxyl radicals. Furthermore, most malignant cells are found to be rich in the heavy chain sub unit

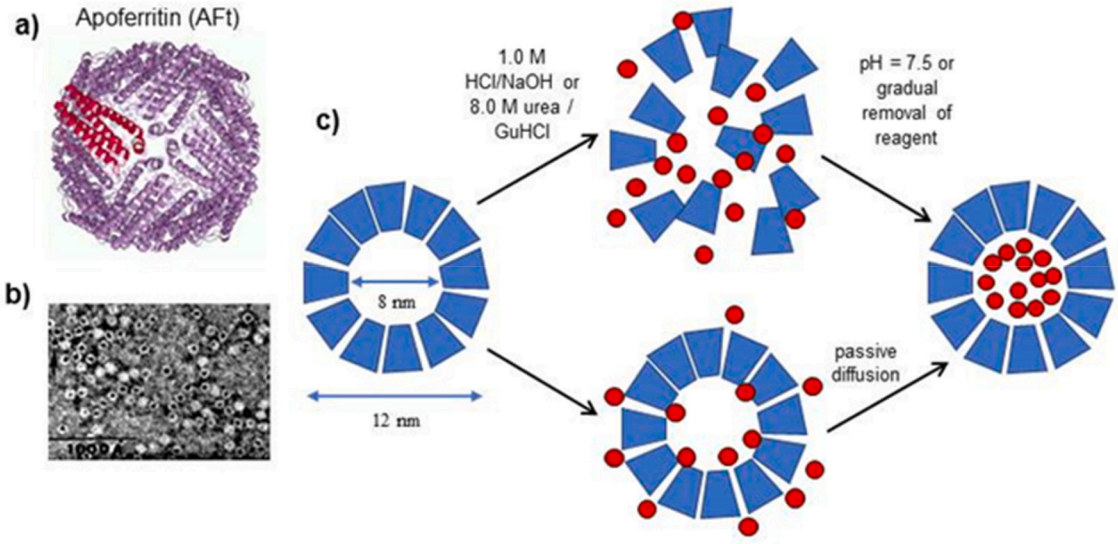

Fig. 1. (a) Image of a spherical shell of protein AFt formed of 24 subunits (peptide chains) arranged in octahedral 4-3-2 symmetry [28]. (b) An electron microscopy image of negatively stained AFt (44). (c) Schematic representation of encapsulation by reassembly (top) and passive diffusion (nanoreactor) methods. AFt has 14 channels formed at the subunit intersections with diameters of 3–4 Å for exchanging their cargo.

[58], since iron is required for many fundamental cellular processes such as for cellular growth, proliferation, regulation of cell cycle and regulation of signalling pathways in order to sustain tumour growth [59].

Moreover, AFt can be easily modified by genetic or chemical manipulation. For instance, the surface of AFt has many amine functional groups that can be modified with targeting ligands such as targeting peptides, aptamers, viral antigens, affibodies, small molecules such as folic acid and fluorescent proteins [60,61]. Another feature of AFt, supporting its use as a nanocarrier is that it remains stable up to high temperatures, for example 80 °C. The light chain subunit binds to scavenger receptor class A member 5 (Scara-5) receptor, while, as alluded to, the heavy chain subunit of AFt binds specifically to human transferrin receptor-1 (TfR1) (also named as cluster of differentition (CD) 71). This gives rise to an intrinsic tumour cell active targeting ability for AFt [60,62]. TfR1 is an integral membrane homodimeric glycoprotein and it is expressed on the surface of cells based on their need for iron. Tumour cells of many cancer phenotypes exhibit upregulated TfR1 expression, corresponding to their increased demand for iron due to rapid proliferation, and high metabolism compared to their non-malignant counterparts. Internalisation of iron is mediated by clathrin coated pit-dependent endocytosis [38]. Ferritin usually binds to a protein substance called transferrin to transport iron to red blood cells. However, it should be noted that the binding epitopes on the heavy chain sub unit are different from the binding epitopes on transferrin; therefore, binding of heavy chain sub unit to TfR1 would not be inhibited by endogenous transferrin [63].

When functional iron depletion occurs, more TfR1 is shown to be present on cell surfaces. For instance, TfR1 is abundantly present in tumour cells, hepatocytes, BBB capillaries and also on neurons for the transportation of iron into the brain [64,65]. As mentioned earlier, the BBB is a highly selective membrane barrier between the central nervous system and blood capillaries, therefore, delivery of drugs into the central nervous system via the blood circulation is challenging. [66]. However, an ideal nanocarrier should be able to penetrate the BBB and target the cells of interest. In this context, AFt becomes beneficial as it can bind to cells which express TfR1 and deliver drugs into the central nervous system which provides a natural pathway to target the affected brain and spinal cord, especially tumours originating from these organs [67,68]. Further, AFt can exploit the EPR associated with the tumour microenvironment - a passive targeting process. This is because the vasculature resulting from tumour angiogenesis within a tumorous zone is porous; thus AFt with its nano size is able to leak into tumour tissue. In addition, dysfunctional lymphatic drainage associated with the tumours leads to drug retention and enhanced intracellular drug concentrations within a tumour, thereby improving the therapeutic efficacy [51]. Furthermore, the tumour microenvironment exhibits lower extracellular pH than normal tissues while the intracellular pH of cells within normal and tumour cells is similar. The overall pH within a tumour environment might be ~6.9 whereas normal cells will have a pH range between 7.2 and 7.6. This more acidic environment develops within tumour cells when increased glucose breaks down, resulting in significant production of lactate and H+ which is transported to the extracellular environment. Further, tumour cells reprogramme energy metabolism to aerobic glycolysis contributing to an acidic microenvironment [69,70]. Therefore as a result of this pH discrepancy, the encapsulated drug may preferably be released within this more acidic tumour microenvironment [71]. Due to these properties of the tumour and AFt, this protein represents an excellent platform for delivering guest molecules.

4. Applications of apoferritin in oncology

To date, AFt has been used to encapsulate a number of therapeutic anticancer agents as described below. Therapeutic activity of encapsulated agents could be affected by the origin of apoferritin as well as its size. Hence, we review some selected examples of anticancer activity of agents encapsulated within synthetic human AFt, horse spleen AFt and Dps capsules.

4.1. Encapsulation of agents using human AFt

Human AFt, as a drug delivery capsule, could offer two major benefits: a reduced immune response and opportunities for engineering the composition of subunits for selective tumour-targeting. Heavy chain human apoferritin is particularly promising for targeting of specific receptors, as we discuss below.

In a previous study the anticancer drug gefitinib was encapsulated (446.9 g/mol) within human heavy chain AFt. Gefitinib is an epidermal growth factor receptor (EGFR) inhibitor, administered mostly for non-small cell lung cancer and certain breast cancers. The diffusion method for encapsulation was adopted where ~10 molecules of drug were encapsulated within the cavity [72,73]. SDS-PAGE and TEM confirmed the stability and structural integrity of the encapsulated agent. SDS-PAGE revealed a band comparable to that of AFt only, indicating that the AFt protein structure and charge remained unchanged after encapsulation; additionally, TEM depicted an intact AFt shell. Interestingly, drug encapsulation into human AFt resulted in enhanced gefitinib activity with a longer exposure time. Accordingly, significant activity-enhancement was reported in HER2-overexpressing SKBR3 breast cancer cells, when exposed to gefitinib-AFt for 120 h (gefitinib-AFt, concentration causing 50% cell growth inhibition (GI_{50}) = 0.52 μM; free gefitinib GI_{50} = 1.66 μM), compared to a shorter exposure time (72 h) (gefitinib-AFt GI_{50} = 1.44 μM; free gefitinib GI_{50} = 0.94 μM). The results illustrated sustained release of drug from AFt. Clonogenic assays further provided evidence supporting continued release of gefitinib, which is a feature of a successful nanotechnology drug delivery system. Further, the authors revealed that AFt alone was non-toxic to cells. Moreover, by utilising the fluorescent property of gefitinib, it was demonstrated by confocal microscopy that gefitinib-AFt could be internalised by certain tumour cells such as SKBR3 cells, due to the presence of ferritin binding receptors - TfR1 and enhanced endocytosis of AFt, thus corroborating human AFt as a suitable drug delivery system (Fig. 3) [53].

Falvo et al. used a genetically modified version of human heavy chain AFt (The-05), to increase selectivity for cancer cells over healthy cells. This particular version is only activated by specific matrix metalloproteinases (MMP 2/9) expressed in the tumour microenvironment. It can extend protein half-life in the bloodstream as compared to native AFt due to the masking polypeptide present on the AFt surface. It is also able to recirculate until it binds to highly-expressing TfR1 cells at tumour sites. The AFt cavity in this study was loaded with ~80 molecules of Genz-644,282 (407.4 g/mol), which was named as 'The-0504' after encapsulation. Genz-644,282 is a non-camptothecin topoisomerase I inhibitor under clinical evaluation. DNA topoisomerases are an important target for many antitumour agents. Topoisomerase enzymes catalyse changes in DNA topology, unravelling the DNA double helix that occurs as a result of DNA transcription and replication. Non-camptothecin topoisomerases I inhibitors are able to inhibit topoisomerase catalytic activity in dividing cancer cells [74]. The drug was encapsulated by the pH disassembly/reassembly process. Methods such as SDS-PAGE, SEC, DLS (AFt outer diameter 17.0 ± 0.7 nm), and TEM (AFt outer diameter 19.7 ± 1.5 nm) endorsed that the overall protein structure and the assembly of AFt was not affected after encapsulation of the drug. Promising results were obtained in preclinical *in vitro* assays: a half maximal inhibitory concentration (IC_{50}) of 21 nM for the encapsulated agent, (free drug IC_{50} = 160 nM) for HT-29 colon cancer cells; and an IC_{50} = 35.9 nM for the encapsulated agent, (free drug 365.4 nM), for HPAF II pancreatic cells were noted, demonstrating good TfR1-mediated uptake by cancer cells. Further, striking results were obtained for HPAF II cancer mice models *in vivo*, where long-term regression was shown in all the established tumours with a tumour growth inhibition of 94% compared to mice given free drug (tumour growth inhibition = 53.3%). Moreover, all animals treated with AFt-

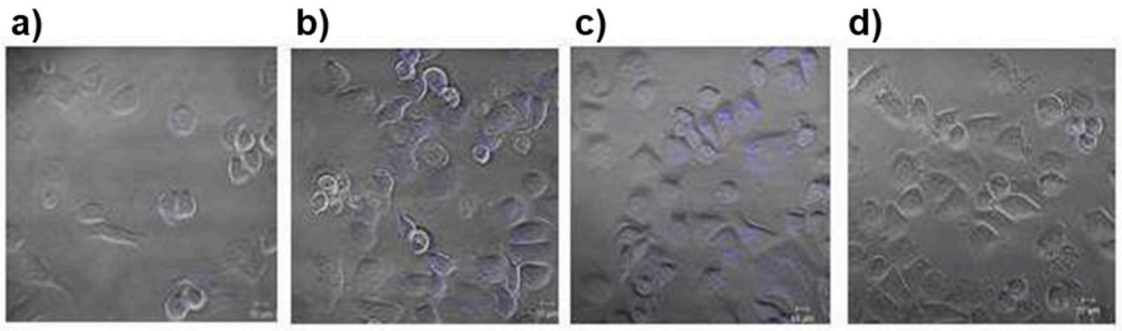

Fig. 3. Confocal microscopy images of SKBR3 breast cancer cells, (a) control cells, and cells following exposure to (b) gefitinib alone, (c) gefitinib-AFt and (d) human heavy chain AFt alone showing cellular uptake and internalisation of gefitinib after 24 h exposure. SKBR3 cells were treated with gefitinib-AFt (5 μM), or gefitinib alone (5 μM). Representative images of trials ≥3, (n = 2) [53].

encapsulated drug survived the entire study period (50 days) compared to animals treated with free drug (38 days), and the mice treated with the encapsulated agent did not show any abnormal behaviour or loss of body weight during the treatment period [75]. Furthermore, in a more recent study it was found that the encapsulated agent, led to long term regression in aggressive solid tumours, such as PaCa44 (pancreatic cancer), MDA-MB-231 (triple-negative breast cancer) and HepG2 (liver cancer) in tumour-bearing mice with 100% values for both tumour growth inhibition and overall response rate. Interestingly, these researchers showed that The-0504 was able to outperform nab-paclitaxel (Abraxane) as well, which is an albumin-bound nano-therapy approved for clinical use, and currently used as standard of care in the treatment of metastatic pancreatic cancer. The enhanced efficacy shown in this study could be due to The-0504's remarkable selectivity for cancer cells that highly express TfR1, and the MMP 2/9 targeting strategy in the tumour environment that eliminates unwanted side effects and lengthens blood half-life [76].

Paclitaxel (853.9 g/mol), is a tetracyclic diterpenoid chemotherapeutic agent originally derived from the bark of the Pacific yew tree, *Taxus brevifolia*. It is given for several cancers in the clinic such as breast, non-small cell lung cancer, pancreatic cancer, ovarian, head and neck, oesophageal, prostate and bladder cancers [77]. However, many agents are unable to penetrate the BBB efficiently, including paclitaxel, which is expected to be highly effective against gliomas if it can traverse the BBB. Glioma cells have depicted enhanced (≥100-fold) TfR1 expression compared to normal cells and TfR1-mediated endocytosis has been a popular choice for transporting pharmaceutical agents into the brain. Thus, Liu et al., encapsulated ~60 molecules of paclitaxel in human heavy chain AFt via the pH disassembly/reassembly strategy. The morphology of the paclitaxel-AFt complex was investigated by TEM and DLS. TEM depicted that the structure was intact, while DLS showed that the outer diameter of paclitaxel-AFt was slightly increased (15.4 nm) after encapsulation, which is observed in most studies. This group of scientists showed that the presence of transferrin could not inhibit the binding between AFt and TfR1 by a competitive binding assay, thus, transferrin in the bloodstream is unlikely to affect AFt attachment to the BBB. They then evaluated the BBB penetration ability by paclitaxel-AFt. High levels of paclitaxel-AFt uptake (20-fold) were found by a co-culture model of bEnd.3 (mouse brain endothelial) and C6 (rat glioma) cells that mimicked the BBB where paclitaxel-AFt was labelled with Cy5-NHS ester dye for visualisation. They also observed stronger fluorescence and deeper penetration of paclitaxel-AFt in a C6 glioma spheroid model compared to free Cy5, which might be due to high affinity to glioma cells. Further, the paclitaxel-AFt complex demonstrated slightly higher potent antiproliferative effects against C6 cells (IC_{50} = 2.2 μg/ml) compared to free drug (IC_{50} = 3.1 μg/ml) by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Similarly, paclitaxel-AFt labelled with Cy5 treated C6 glioma tumour-bearing mice showed stronger fluorescence around the brain region after 1 h of drug administration, which confirmed that paclitaxel-AFt could traverse the BBB and accumulate in the brain. They also measured the amount of encapsulated agent in organs isolated from mice 2 h after intravenous injection. Intriguingly, they found a 10-fold increase of paclitaxel in the brain when it was encapsulated within AFt, which could be due to enhanced penetration of the BBB. The paclitaxel-AFt complex also increased mouse median survival time to 30 days, which was significantly longer than mice treated with free drug (14 days) and physiological saline (13 days). Moreover, paclitaxel-AFt did not show a reduction in mice body weight compared to mice treated with physiological saline, which might be due to lower toxicity of the encapsulated agent. Likewise, no significant hyperaemia, necrosis, or inflammation were observed in other organs of mice when treated with paclitaxel-AFt, which may be because of the enhanced tumour targeting ability of AFt and decreased side effects [63].

Bellini et al. encapsulated doxorubicin (543.5 g/mol), an antitumour anthracycline antibiotic chemotherapeutic agent, within human heavy chain AFt using the pH disassembly/reassembly route. Around 28 molecules of the drug have been loaded within the cavity. There have been many studies describing encapsulation of this agent in various nanoparticles; and in fact it was the first nanoparticle-based treatment to be approved for cancer by the U.S. Food and Drug Administration (FDA) in 1995, which was named as 'Doxil', a formulation of doxorubicin delivered via liposome nanoparticles which showed fewer side effects compared to the conventional agent [78]. Doxorubicin is administered to treat bladder, ovarian, breast cancers, lymphoma, and acute lymphocytic leukaemia. With the use of AFt encapsulation, the effects of the drug may endure longer in the body by selective unloading at the affected cancer sites [79]. Thus, this group of researchers showed reduced cell survival (< 5%) in Hela (cervical cancer) cells when treated with 0.1 μM of doxorubicin-AFt complex compared to cells treated with free doxorubicin (0.1 μM) (< 20%) at 72 h. Hela cells also engulfed the encapsulated agent more rapidly and efficiently compared to free drug as observed by confocal microscopy where doxorubicin-AFt was detected in the nucleus after a short duration of treatment (3 h), as demonstrated by analysis of doxorubicin fluorescence spatial distribution. This demonstrates that AFt is able to translocate into the nucleus and deliver DNA interacting molecules that can exert a specific intra-nuclear stimulus. As such, the encapsulated agent displayed enhanced DNA damage compared to the free agent in cervical cancer cells [80]. More recently, the same group showed that female mice bearing HER2 positive breast cancer tumours, treated with a combination of doxorubicin-AFt and Trastuzumab (monoclonal antibody used to treat HER2 positive breast cancer; 145,531.8 g/mol), demonstrated tumour regression, while agents alone (doxorubicin-AFt or Trastuzumab) only slowed down tumour growth. Interestingly, this agent combination also showed lower cardiotoxicity in mice heart tissue despite the amount of drug in heart being the same as free drug, which was remarkable. Cardiotoxicity has been a main side effect associated with both agents in the clinic and

many clinical trials have shown that these two agents should not be co-administered although they result in significant antitumour efficacy. Thus, clinical translation of doxorubicin-AFt and Trastuzumab in combination may address this major issue [81].

The results with human AFt reported to date are promising; however the composition of human protein significantly affects its uptake and could also affect the drug retention and release. This makes comparison of the activity challenging as well as presenting difficulties for independent trials and reproducibility of results. Further studies and development of standard approaches to expression and purification of human AFt are needed to enable systematic studies of their potential as drug delivery capsules.

4.2. Encapsulation of agents using horse spleen AFt

Despite potential benefits of using human AFt, it is not widely available and requires complex synthetic biology for expression and purification [82,83], hence commercially available proteins are widely used. We note, that strategies developed using horse spleen AFt are easily transferable onto proteins of different origin, and, as we discuss below, the *in vitro* therapeutic results are comparable.

The benzothiazole pharmacophore has proven to be important in cancer where it demonstrates potent and selective antitumour activity *in vitro* and *in vivo*. The fluorinated benzothiazole analogue 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole (5F 203) has been shown to induce aryl hydrocarbon receptor (AhR) signalling. This induces cytochrome P450 (CYP) 1A1 expression that catalyses metabolic activation of the benzothiazoles, producing lethal DNA adducts in sensitive cancer cells. Breen et al., encapsulated 5F 203 (258.3 g/mol) in horse spleen AFt, by passive diffusion and also by exploiting the AFt disassembly/reassembly method. Approximately 70 molecules of 5F 203 were encapsulated within the AFt cage by the diffusion method. Interestingly, this group investigated both the pH method as well as the urea method when exploiting the disassembly/reassembly route. The results indicated that both methods could only encapsulate ~25 molecules of drug within AFt compared to the passive diffusion route. This could be due to therapeutic agents losing their structure and function at extreme pH levels or in the presence of denaturing agents. Moreover, the protein cage could be damaged at these extreme pH levels, leading to low encapsulation efficiency, although many studies have effectively used this method [38]. ToF-SIMS confirmed that 5F 203 was exclusively within the AFt cavity and not bound to the surface. They also encapsulated the lysylamide prodrug of 5F 203 - Phortress (386.5 g/mol) via the diffusion method, where ~130 drug molecules were entrapped per AFt cage. Remarkably, it was found that the encapsulated benzothiazoles were more potent depicting nanomolar activity, than free agents, in most cell lines tested such as IGROV-1 (ovarian cancer), TK-10 (renal cancer), MKN-45, KATO-III and NCI-N87 (gastric cancer cell lines) with $GI_{50} < 0.1$ μM. In contrast, nontumourigenic MRC5 foetal lung fibroblast cells depicted $GI_{50} > 50$ μM, illustrating selective activity in benzothiazole-sensitive cancer phenotypes [54]. Further, the same group encapsulated structural analogues of benzothiazoles (GW 610 and GW 608) in horse spleen AFt. These analogues are also AhR ligands. They entrapped >190 drug molecules of GW 610 per AFt cage and > 110 molecules of GW 608 per AFt cage via the diffusion method. It was shown that encapsulated GW 610 was ~50 times more potent compared to the drug alone especially in colorectal cancer cells: KM12 adenocarcinoma cell line (GW 610-AFt $GI_{50} = 0.45$ μM; free GW 610 $GI_{50} = 22.0$ μM). GW 610-AFt was able to completely abolish KM12 colony formation, indicating that the encapsulated formulations possess high cytotoxic properties and inhibit formation of progeny cell colonies. GW 608 was less potent in all cell lines tested, which could be due to reduced lipophilicity and lower affinity for AhR. However, GW 608 derivatives when conjugated with amino acids, illustrated high encapsulation efficiency with >308 molecules of GW 608-Lys per AFt cage. Upon encapsulation via the diffusion route, GW 608-Lys conjugate depicted significant enhanced potency (~1000 fold) against KM12 cells, compared to free drug (GW 608-AFt $GI_{50} = 0.13$ μM; free GW 608 $GI_{50} > 100$ μM) which was noteworthy [51]. Despite promising results on therapeutic activity of benzothiazoles, significant differences in encapsulation rate are observed. Note, that uptake is defined by the capsule, while therapeutic activity is strongly affected by the amount of cargo. Hence, systematic studies of the effect of loading efficiency of anticancer activity are needed to advance these agents into the clinic.

Cysteine proteases are known as protein processing enzymes in mammals where they perform key roles in cell survival and proliferation. However, they also have a role in tumourigenesis, causing protein degradation and promoting invasive tumour growth [84]. Certain aggressive cancers such as pancreatic and colorectal carcinomas express cathepsin L, a lysosomal cysteine protease, which acts as an overexpressed marker. Therefore, cysteine protease inhibitors are currently considered as highly selective novel anticancer drug leads. A recent study showed that novel dipeptidyl nitrile-based cysteine protease inhibitors (Neq0551, Neq0554 and Neq0568) could be encapsulated into horse spleen AFt by diffusion. Approximately 117, 105 and 226 of Neq0551, Neq0554 and Neq0568 molecules were encapsulated respectively within the AFt cavity. SDS-PAGE and DLS illustrated that the size and shape of AFt did not change after encapsulation of the drugs. It was also shown that the value of the zeta-potential measured for AFt alone was -8.6 ± 0.8 meV, and was not changed following encapsulation of test agents confirming that agents are encapsulated within the AFt cavity. Interestingly, AFt-encapsulated Neq0554 demonstrated 3-fold enhanced activity *in vitro* in both MiaPaCa-2 (pancreatic cancer) and HCT-116 (colorectal cancer) cells, a possible consequence of enhanced TfR1-mediated endocytosis. Out of the 2 cell lines, MiaPaCa-2 cells were more sensitive to the encapsulated agent $GI_{50} = 79.5$ μM compared to free drug ($GI_{50} = 230.7$ μM). Selectivity for cancer cells had also been confirmed by comparing encapsulated Neq0554 activity to non-tumourigenic MRC5 cells ($GI_{50} > 200$ μM). This group has detected TfR1 protein expression of HCT-116 and MiaPaCa-2 cells by western blot analysis; in contrast, TfR1 levels had been undetectable in non-tumuorigenic MRC5 cells inferring disparity in TfR1 expression between cancer and non-cancer cells. Further, MiaPaCa-2 colony formation was dramatically impeded (>60%) by the encapsulated agent, which might be due to enhanced cellular uptake. Furthermore, exposure of HCT-116 cancer cells to Neq0554-AFt showed enhanced pre-G1 populations, indicating apoptosis, and a reduction in DNA synthesis (S) phase (by ~15%) in cell cycle analyses (Fig. 4), suggesting reduced DNA replication and cytostasis after treatment with the encapsulated agent. Interestingly, when HCT-116 and MiaPaCa-2 cells were exposed to free Neq0554 (50 μM) as well as the encapsulated agent (50 μM), total cathepsin L activity was inhibited effectively by Neq0554-AFt which was long lasting, compared to free drug. This could be due to efficient TfR1-mediated uptake of the encapsulated agent and sustained release of cargo in acidic lysosomes of cancer cells [52]. Similar to the study of benzothiazoles, interaction of the Neq-agents with the protein exterior and effect of drug loading and retention are needed.

The potential of AFt for convection enhanced drug delivery in brain cancers was explored with AFt-encapsulated temozolomide (194.1 g/mol) with the ultimate objective *in vivo* of achieving enhanced BBB penetration. Glioblastoma multiforme (GBM) is a grade IV astrocytoma, a rapidly-growing aggressive glioma that develops in the central nervous system. The current standard of care for GBM consists of surgical resection, followed by radiotherapy with concurrent temozolomide chemotherapy. However, poor accumulation and resistance to temozolomide is shown in the brain, possibly due to active drug efflux transport proteins such as P-glycoprotein, short plasma half-life, overexpression of *O6*-methylguanine-DNA methyltransferase (MGMT) or deficiencies in the DNA mismatch repair pathways [85]. Horse spleen AFt has been used by loading ~520 drug molecules per AFt cage via the diffusion route. The integrity of the AFt cage following drug encapsulation was confirmed by DLS, and SDS-PAGE. Enhancement of antitumour activity

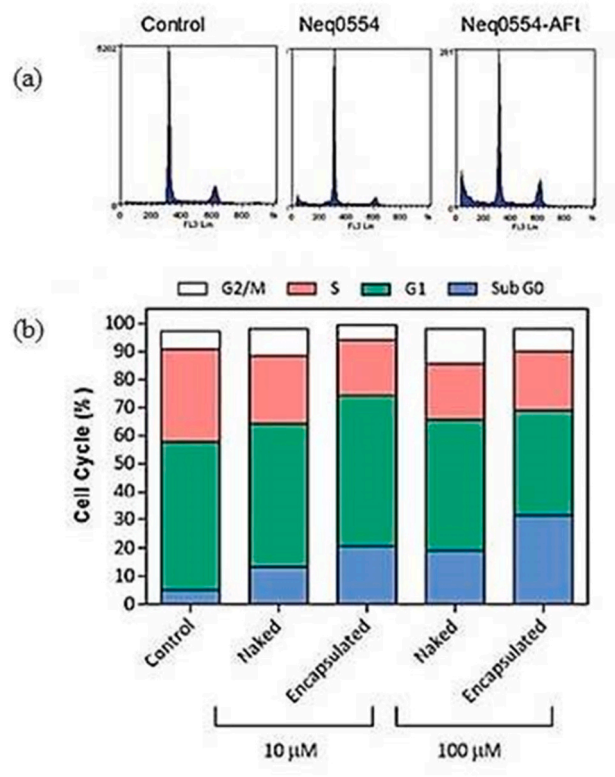

Fig. 4. (a) Represntative cell cycle profiles of HCT-116 colorectal cancer cells and (b) histogram of cell population distribution following cell treatment with Neq0554 alone and Neq0554-AFt for 48 h. Trials ≥3, ($n = 2$). Increased pre-G1 population was observed for Neq0554-AFt treated cells, compared to control [52].

in U373V (MGMT low) and U373M (MGMT positive and temozolomide resistant) GBM cell lines was reported with the encapsulated agent, with GI_{50} values <1.5 μM; compared to free drug (GI_{50} values of 35 and 376 μM were shown for free temozolomide in U373V and U373M cells, respectively), supporting the hypothesis that temozolomide-AFt overcomes tumour resistance mediated by MGMT, in MGMT positive GBM cell lines. Indeed, U373M cells showed a remarkable 532-fold enhanced activity when treated with temozolomide-AFt. It was also observed that U373M cells were less able to survive temozolomide-AFt challenge and form progeny cell colonies, revealing ~76% inhibition of colony formation (Fig. 5).

Further, cell cycle progression was analysed after 72 h of exposure of cells to temozolomide and temozolomide-AFt where both U373V and U373M cells expressed greater S and G2/M phase arrests following exposure to temozolomide-AFt. Subsequently, whether cell cycle arrest translated to higher DNA damage was assessed; indeed, increased *O6*-methylguanine levels and subsequent γH2AX foci were observed following treatment of U373V (1.2-fold more) and U373M (1.4-fold more) cells with temozolomide-AFt compared to free agent indicative of DNA double strand breaks. This group of researchers also encapsulated, via the diffusion route, N3P, an analogue of temozolomide where N3-methyl of temozolomide has been replaced with a propargyl moiety. A similar number of drug molecules were encapsulated (~525). Enhanced activity with lower GI_{50} concentrations were reported in both GBM cell lines ($GI_{50} < 0.25$ μM) for N3P-AFt. Enhanced activity of these encapsulated agents might be due to a different mode of cellular uptake via TfR1 recognition, where AFt rapidly enters the cells and accumulates in high levels within lysosomes, which might overwhelm MGMT and also assist evasion of P-glycoprotein efflux in cells [86]. Promising results were demonstrated *in vivo*, with N3P-AFt showing enhanced activity in spheroids and rat models [87]. While convection enhanced drug delivery is of potential interest, the pharmacokinetics of the AFt encapsulated agents needs to be explored in detail. The potential of AFt nanocages to pass the blood brain barrier (BBB) [67,88] could also be explored and will provide more favourable options for brain cancer treatments.

Alqaraghuli et al. tested epirubicin (543.5 g/mol) loaded horse spleen AFt against MCF-7 breast cancer cells to decrease toxicity to normal cells and improve targeted drug delivery [89]. The encapsulation was carried out by the pH disassembly/reassembly method; a ratio of 1:100 of AFt:epirubicin molecules was adopted. Epirubicin is an anthracycline topoisomerase inhibitor administered mostly for breast cancer [90]. The surface of AFt was modified using folic acid as a targeting ligand for optimal targeting of breast cancer cells since it enhances the cellular uptake efficiency via folic acid-receptor-mediated endocytosis. Folic acid is necessary for DNA synthesis, and most tumour cells overexpress folic acid receptors. These researchers used several methods to determine the stability of AFt after encapsulation, such as UV–vis spectroscopy, fluorescence spectroscopy, and TEM; it was found that the drug molecules were encapsulated within the AFt cavity and the surface of the AFt was modified by a targeting ligand- folic acid. Interestingly, the folic acid-epirubicin-AFt complex showed enhanced toxicity (15% cell viability) against MCF-7 cells compared to epirubicin-AFt only (25.83% cell viability) and the free drug (35.33% cell viability) after 72 h exposure. This may be due to the presence of folic acid on AFt which enhances the delivery to malignant cells mediated via folic acid receptors. It was also found that 97.5% of drug was released from the AFt cage at pH 5.0 during 42 h compared to pH 7.4 which might be preferential in a slightly more acidic tumour environment. The release had been rapid in the initial few hours. This could be due to weakening of intermolecular hydrogen bonds between hydrogen of hydroxyl groups in epirubicin molecules and amino groups in the internal structure of AFt, causing numerous drug molecules to release initially. This example demonstrates that surface modification of AFt enhanced targeting to cancer cells [89].

Oxaliplatin-loaded AFt conjugated with panitumumab via a polyethylene glycol linker (AFPO) was designed to target cell lines expressing EGFR [61]. Oxaliplatin (397.2 g/mol) is used to treat colorectal cancers and it is often given in combination with other anticancer agents. Oxaliplatin was encapsulated in horse spleen AFt, by adjusting the pH value to exploit the AFt disassembly/reassembly method. This drug is a platinum-based chemotherapy agent prescribed for colorectal

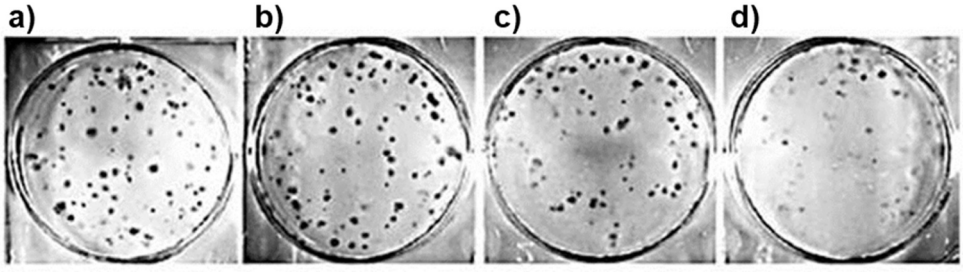

Fig. 5. Representative images of clonogenic assays conducted on U373M GBM cancer cells, exposed for 6 days to (a) media alone (control), (b) AFt 0.057 μM, (c) temozolomide 50 μM and (d) temozolomide-AFt 50 μM. A significant reduction in U373M colony formation (76%) was observed following temozolomide-AFt treatment, compared to control. AFt alone had a negligible effect, which demonstrates the biocompatibility of this protein as a drug delivery vehicle. Trials ≥3, ($n = 5$) [86].

cancer. Panitumumab (144,324.1 g/mol) is a human monoclonal antibody targeting EGFR, overexpressed in metastatic colorectal cancer [91]. TEM indicated that oxaliplatin was successfully encapsulated in the AFt cage because of the high electron density observed within the AFt cavity. The AFt diameter was increased from 13 to 19 nm due to the conjugation of panitumumab to the surface of AFt. By fluorescein isothiocyanate (FITC), it was determined that each AFPO nanocage was conjugated to ~2 panitumumab molecules with a conjugation efficiency of 57.5%. AFPO showed high affinity for HCT-116 cancer cells that express high levels of EGFR, and treatment resulted in low cell viability of 19.9% compared to oxaliplatin-loaded AFt after 48 h (33.2%). Free oxaliplatin was extremely toxic to cells with a viability of 3.6% at 48 h, when compared to panitumumab alone - which did not show obvious toxicity (96.1% at 48 h). Interestingly, it was found that the AFPO binding to the EGFR receptor was inhibited once cells were pre-treated with panitumumab antibodies for 1 h prior to addition of AFPO, which confirms specific binding of AFPO to the EGFR receptor. The viability of the HCT-116 cells was then increased from 19.9% to 46.5% after 48 h (Fig. 6).

Further, oxaliplatin is known to damage DNA and induce cell apoptosis, thus it was found that HCT-116 cells exhibited early apoptosis when treated with AFPO nanocages for 24 h (13.3%) and 48 h (15.5%). They also found that AFPO uptake was mediated by the endosomal/lysosomal system where fluorescence intensity was stronger for HT29 cells (moderate EGFR expressing) compared to colon cancer cells SW-620 (low EGFR expressing), which indicated via confocal microscopy, that higher EGFR expression was associated with greater uptake of AFPO. This design released the drugs and suppressed tumour cell growth efficiently together with greater accumulation of AFPO in tumour cells in BALB/c nude mice bearing HCT-116 K-Ras mutant tumours (tumour growth = 205.9 ± 27 mm^3 in AFPO compared to 717.2 ± 263.4 mm^3 in oxaliplatin-loaded AFt only) in 24 days, which might be due to the EPR effect and enhanced targeting ability. Furthermore, AFPO nanocage-targeted therapy led to longer survival duration (>39 days) in HCT-116 tumour bearing mice. Importantly, it was also found that AFPO nanocages caused no obvious damage to normal tissue compared with the tissues in the control group examined in major organs such as the heart, liver, spleen, lung and kidney demonstrating that AFPO is safe. This study demonstrated that combining oxaliplatin and panitumumab into one formulation improves the targeting ability using AFt and could be a promising application in the clinic [61].

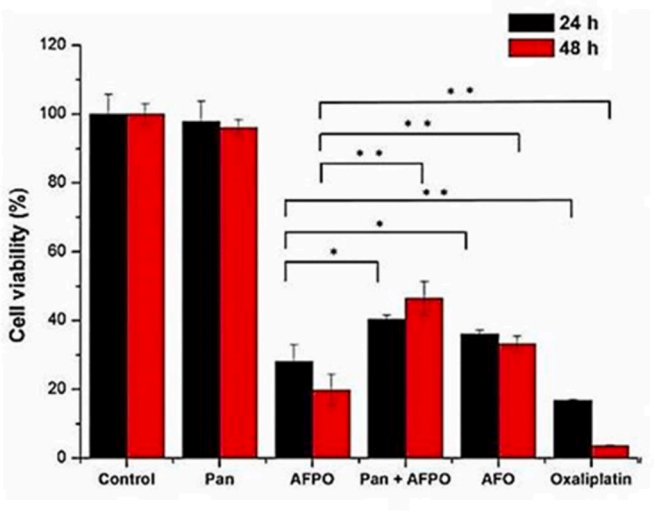

Fig. 6. Histogram of MTT cell viability assay of HCT-116 colorectal cancer cells. All tested agents contained 10 μM of the drug oxaliplatin. Oxaliplatin-loaded AFt conjugated with panitumumab (Pan) via a polyethylene glycol linker (AFPO) showed a cell viability of 28.1% and 19.9% at 24 and 48 h respectively, compared to oxaliplatin-loaded AFt (AFO), which demonstrated a higher cell viability (36.2% at 24 h and 33.2% at 48 h). Free oxaliplatin was toxic to cells compared to Pan which did not show obvious toxicity. Specific AFPO binding to EGFR was investigated by a competitive binding assay, where HCT-116 cells were pre-treated with Pan (67 μM) for 1 h before adding AFPO. The cell viability was increased from 28.1% to 40.3% after 24 h and, from 19.9% to 46.5% after 48 h, which demonstrated specific binding to EGFR had been blocked by pre-treating cells with Pan. Bars represented means ± standard deviation ($n = 3$); * $p < 0.05$, and ** $p < 0.01$ [61].

Natural products also play a key role in cancer management. Natural products have been a source for successful drug development for thousands of years including drugs for cancer [92]. Tumourigenesis is a multistep process which begins with cellular transformation and progresses to a hyperproliferative cancerous lesion. Chemopreventive agents are found to suppress these transformative processes that initiate carcinogenesis [93]. Mansourizadeh et al., encapsulated quercetin (Que) (302.2 g/mol) and curcumin (Cur) (368.3 g/mol) in horse spleen AFt (Que-Cur-AFt) by the pH disassembly/reassembly process. The entrapment of the compounds within AFt was followed by size exclusion chromatography that compared the curves acquired at 280 nm, 370 nm and 430 nm, corresponding to the maximum absorbance of the AFt protein, Que. and Cur, respectively. These results showed that the compounds were unequivocally localised within the AFt cavity. A low half maximal effective concentration (EC_{50}) (11 μM), was found for the combination of Que-Cur-AFt (which was assembled in 1:1 ratio) compared to the Que-Cur free combination (EC_{50} = 28.6 μM) and the agents alone (EC_{50} = Que. >100 μM and Cur >58.5 μM) against MCF-7 breast cancer cells. Further, they found that the Que-Cur free combination demonstrated a similar EC_{50} (20.8 μM) to MCF-7 cells for MCF10A, which is a non-tumourigenic breast epithelial cell line, while in any other formulation of Que. and Cur the EC_{50} was >100 μM for MCF10A cells; whereas encapsulation of the two compounds exerted synergistic potent growth inhibitory effects that were specific to MCF-7 cancer cells. They also showed an increase in late stage apoptosis, intracellular ROS levels and internalisation of Que-Cur-AFt, compared to free combination and agents alone in MCF-7 cells at a concentration of 2.74 μM Cur and 3 μM Que. for all agents. Also, sequestration of these compounds within AFt prevents Que. and Cur binding to serum proteins, which may increase the amount of bioactive compounds available for tumour cell internalisation. Therefore, this study showed that encapsulation of Que. and Cur in AFt, increased the synergistic cytotoxicity of the agents in combination in cancer cells [94]. Flavonoids such as Que. and Cur have received considerable attention lately as chemopreventive food supplements that lower the risk of certain cancers such as breast and colon. They might also have a potential use in the management of cancer in addition to primary therapy, due to modulating biological events in cancer such as cell apoptosis [95]. Intriguingly, these studies demonstrate that horse spleen AFt as a useful carrier for therapeutics as it has shown enhanced activity in sensitive cancer cell lines over free drugs similar to human AFt.

To explore the potential of AFt for theranostic applications, Bradshaw et al., encapsulated lead sulphide quantum dots (PbS QDs) within horse spleen AFt. PbS QDs have optical emission in the near-infrared wavelength range of low absorption of biological tissues, and could offer benefits for non-invasive deep tissue imaging [96]. Semiconducting colloidal QDs are nanocrystals with a promise for a range of applications from biological imaging as fluorophores to cancer therapeutics, becoming an excellent theranostic application in cancer. As such this group of researchers investigated the effect of PbS-encapsulated AFt in colorectal cancer cell lines. Encapsulation provides the QDs a water-soluble hybrid construct with stable and tunable fluorescence emission at wavelengths >1000 nm, which allows deep tissue imaging, and also addresses the challenges with its extreme toxicity during *in vivo* imaging. The pH-dependent reassembly method was used to encapsulate PbS QDs in horse spleen AFt (PbS-AFt). TEM images revealed an electron-dense central core with PbS, which showed the presence of nanoparticles within AFt. The existence of the PbS nano-

crystals within AFt was also confirmed by energy dispersive X-ray (EDX) analysis, which revealed characteristic Pb and S peaks. They found that two colorectal carcinoma cell lines demonstrated a GI_{50} value of ~70 μg/ml compared to non-tumourigenic HMEC-1 cells (immortalised human microvascular endothelial cells), which showed ~10-fold resistance (GI_{50} = ~800 μg/ml). AFt alone had no effect on cells. The encapsulated agent also demonstrated high levels of pre-G1 populations in cell cycle analyses and early as well as late stage apoptosis with 70 μg/ml of PbS-AFt in both cell lines. When HMEC-1 cells were exposed to 70 μg/ml of PbS-AFt, cells displayed neither perturbed cell cycle nor induced apoptosis which depicted outstanding selectivity to cancer cells. Strikingly, a low concentration of PbS-AFt (0.1 x GI_{50}; 7 μg/ml) was able to completely inhibit colon cancer cell colony formation of both cell lines demonstrating enhanced cytotoxicity. HCT-116 cells were challenged with the encapsulated agent at concentrations of GI_{50} (70 μg/ml) and 2× GI_{50} (140 μg/ml) for just 1 h, which showed enhanced reactive oxygen species (ROS) by 29% and 70%, respectively; compared to untreated cells. This result may confirm that PbS-AFt nanoparticles evoke cytotoxicity in cancer cells triggering apoptosis via a mechanism that involves PbS-induced generation of ROS. Evidence presented herein corroborated previously reported data by Turyanska et al. which is discussed under Section 4.3 that PbS-AFt evoked cancer cell specific apoptosis through elevation of ROS within breast cancer cells as well following TfR1-mediated endocytosis. These researchers also determined whether PbS-AFt could be tolerated *in vivo*. They tested PbS-AFt in female athymic mice and found that the agent was well tolerated by the animals. No adverse side effects, weight or behavioural changes were observed during the study period (15 days). These encouraging data suggest that this application should be evaluated further for future theranostic clinical use in cancer [97].

4.3. Encapsulation of agents using horse spleen AFt and human AFt

AFt can be useful for theranostic applications where it can act as a potential diagnostic as well as a therapeutic tool, however effect of the origin of protein capsule needs to be explored and can enable image-guided therapy, which may ultimately lead to personalised therapy [98–100]. Turyanska et al. incorporated PbS near-infrared QDs into both human and horse spleen AFt. Both the pH-dependent reassembly and the diffusion processes were incorporated to encapsulate PbS nanocrystals inside the AFt shell [101,102]. Intriguingly, they did not find any significant differences in the optical and morphological characteristics of PbS-AFt composites synthesised using either the pH-dependent re-assembly or diffusion routes. However, according to them the pH dependent re-assembly route might be easily implemented to synthesise hybrid structures based on other preformed and/or commercially available nanoparticles. Semiconductor PbS QDs are alternatives to organic dyes where they show high signal brightness, and photostability. They have also been shown to possess cytotoxic activity. Hence, they are good candidates for medical imaging as well as therapy as mentioned in Section 4.2 [102,103]. Extensive studies had been carried out to investigate the cytotoxic activity of PbS-AFt of both horse spleen and human AFt, on breast cancer cell lines; MDA-MB-468 and MCF-7 compared to non-tumourigenic MRC5 fibroblasts. Breast cancer cells displayed a $GI_{50} \leq 0.2$ mg/ml for PbS-AFt using both human and horse spleen AFt, demonstrating that the cytotoxic effects induced by both horse spleen and human AFt are very similar. MRC5 cells retained much higher viability (>70%) compared to cancer cells. In a more recent study, it was found that, PbS-AFt increased pre-G1 populations in cell cycle analyses, triggering apoptotic cell death associated with increased ROS levels at concentrations >0.2 mg/ml in MDA-MB-468 and MCF-7 cancer cells. Also it should be noted that there was no phase-specific cell cycle arrest following cell exposure to PbS-AFt. Pre-G1 populations represent sub diploid events consistent with apoptosis-associated DNA fragmentation which is a consequence of caspase activity. Indeed, when MDA-MB-468 cells were subjected to apoptotic analysis, exposure of cells to 1 mg/ml of PbS-AFt for 24 h resulted in 57% of cells in late apoptosis. Furthermore, elevated ROS levels shown by cancer cells might be due to their higher metabolism and genetic instability, resulting in greater susceptibility to ROS imbalance. However, the same agent did not mediate any cellular alterations when tested in non-tumourigenic MRC5 cells ($GI_{50} > 1$ mg/ml) demonstrating that encapsulation of PbS in AFt may facilitate the cellular accumulation of the drug in high concentrations at the tumour site. Furthermore, TEM studies confirmed the cellular localisation of PbS-AFt (Fig. 7), with an observed cellular uptake by endocytosis rather than diffusion in MDA-MB 468 cells. Therefore, it is evident that the presence of the AFt shell enhances the internalisation of PbS into cancer cells, and this selectivity could be further investigated as (a) nanomedicine, and (b) fluorescent labelled markers in imaging studies [102]. A summary of all the above discussed examples could be found in Table 1.

Considering the overall findings of the above mentioned work, it is evident that AFt is a promising nano platform for drug delivery: it is biocompatible, possesses built-in active (TfR1) targeting, and may exploit the EPR effect associated with tumour microenvironments. This evidence suggests that anticancer drug molecules could be encapsulated within AFt successfully and that the encapsulated agents are internalised by cells and animals in preclinical investigations. Whereas use of colloidal semiconductor QDs is of potential interest, concerns over the use of heavy metals remain, and long-term studies on release of heavy metal ions from the QD surface and of clearance of encapsulated QDs from the body are needed. Nevertheless, all encapsulated agents in AFt demonstrated enhanced anticancer activity compared to free agents probably due to increased sequestration by cancer cells in preclinical *in vitro* studies. Further, the AFt cage enabled sustained steady release of the agent over a period of time. *In vivo* studies also showed that AFt is safe and well tolerated by mice and that it increased the survival duration of animals. It should be noted that due to the versatility of these unique nanoparticles, their applications are not limited to cancer treatment, but also useful in the treatment of other diseases, for diagnostics, imaging, delivery of vaccines and for catalysis processes [38].

5. Dps as a delivery carrier and its application in oncology

The biodistribution and uptake on nanoscale materials is strongly affected by their size, hence to explore the effect of protein capsule size we consider Dps protein, which has an outer diameter of 9 nm (compared to 12 nm for AFt).

Kolter and co-workers discovered Dps from starved *E. coli* cells in 1992, not so long ago [104]. They are members of the ferritin

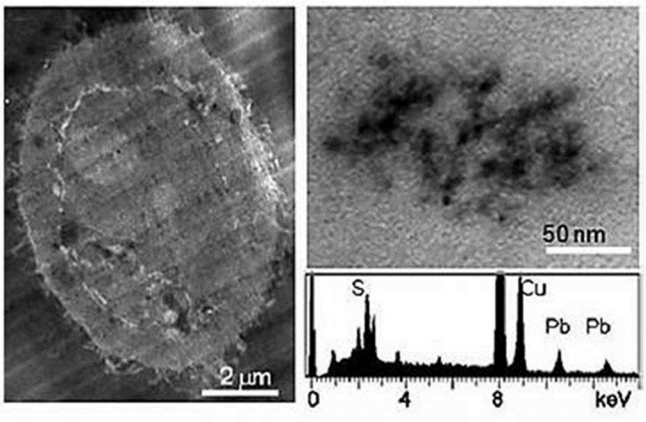

Fig. 7. A TEM image of a MDA-MB-468 breast cancer cell cross-section treated with 1 mg/ml of horse spleen PbS-AFt for 24 h, exhibiting cellular localisation of PbS QDs clusters in the cell cytoplasm, which is supported by the EDX spectra [102].

Table 1
Tumour targeting abilities of anticancer agents when encapsulated within AFt nanoparticles.

Encapsulated agents, number of molecules per AFt and encapsulation efficiency (EE)	Encapsulation method	Tumour models treated	Ref.
Human heavy chain AFt			
Gefitinib: 10 molecules, EE 55%	Diffusion	Breast cancer cells: SKBR3 cells (HER2-overexpressing): $GI_{50} = 0.52 \pm 0.17$ μM (free agent $GI_{50} = 1.66 \pm 0.79$ μM) MDA-MB 231 cells (HER2 negative): $GI_{50} > 25$ μM (free gefitinib $GI_{50} = 19.56 \pm 0.64$ μM)	[53]
Genz-644,282: 80 molecules	pH disassembly/ reassembly	HPAF II pancreatic cancer cells: $IC_{50} = 35.9$ nM (free agent $IC_{50} = 365.4$ nM) Long-term regression in mice models with a tumour growth inhibition of 94%. PaCa44 pancreatic cancer, HepG2 liver cancer and MDA-MB 231 breast cancer tumour bearing mice models: 100% tumour growth inhibition and overall response rate.	[75,76]
Paclitaxel: 60 molecules	pH disassembly/ reassembly	C6 rat glioma cells: $IC_{50} = 2.2$ μg/ml (free agent $IC_{50} = 3.1$ μg/ml); C6 rat glioma tumour bearing mice showed a 10-fold enhancement of agent concentration in the brain for encapsulated agent compared to free drug.	[63]
Doxorubicin: 28 molecules	pH disassembly/ reassembly	Hela cervical cancer cells: Decrease in cell proliferation compared to free agent; HER2+ breast cancer models: A combination of doxorubicin-AFt and Trastuzumab showed tumor regression.	[80,81]
Horse spleen AFt			
5F 203: 70 molecules, EE 47% Phortress (a prodrug of 5F 203): 130 molecules, EE 8% Structural analogues: GW 610: 190 molecules GW 608: 110 molecules GW 608-Lys: 308 molecules	Diffusion	IGROV-1 ovarian cancer: 5F 203-AFt $GI_{50} = 0.039$ μM (free agent $GI_{50} = 12.8$ μM); TK-10 renal cancer: phortress-AFt $GI_{50} = 0.098$ μM (free agent $GI_{50} = 6.3$ μM); MKN-45 gastric cancer: GW 608-Lys-AFt $GI_{50} = 0.21$ μM (free agent $GI_{50} = 22.1$ μM); KM-12 colon cancer: GW 608-Lys-AFt $GI_{50} = 0.13$ μM (free agent $GI_{50} \geq 100$ μM).	[51,54]
Cysteine protease inhibitor Neq0554: 105 molecules, EE 51%	Diffusion	HCT-116 colon cancer: $GI_{50} = 131.0$ μM (free agent $GI_{50} = 358.6$ μM) MiaPaCa2 pancreatic cancer: $GI_{50} = 79.5$ μM, (free agent $GI_{50} = 393.0$ μM).	[52]
Temozolomide: 520 molecules, EE 84% N3P (analog of temozolomide): 525 molecules, EE 71%	Diffusion	U373V and U373M glioblastoma multiforme cell lines: temozolomide-AFt: $GI_{50} < 1.5$ μM (free agent $GI_{50} > 30$ μM) N3P-AFt: $GI_{50} < 0.25$ μM.	[86]
Lead sulphide (PbS) QDs: 1 quantum dot per AFt	Diffusion and pH disassembly/ reassembly	HCT-116 colorectal cancer cells: $GI_{50} \sim 70$ μg/ml; well tolerated in tumor models. MDA-MB-468 and MCF-7 breast cancer cells: $GI_{50} \leq 0.2$ mg/ml.	[97,101,102]
Epirubicin: EE 86%	pH disassembly/ reassembly	MCF-7 breast cancer cells: Folic acid modified AFt for targeting of cancer cells: reduced viability 15% (free agent 35%).	[89]
Oxaliplatin conjugated with panitumumab: EE 88%	pH disassembly/ reassembly	HCT-116 colon cancer cells (overexpression of EGFR): Reduced viability 19.9%; SW-620 colon cancer cells (low EGFR expression): no effect on cell viability.	[61]
Quercetin with Curcumin: Molar ration 1:1	pH disassembly/ reassembly	MCF-7 breast cancer cells: $EC_{50} = 11$ μM (free agents $EC_{50} = 28.6$ μM).	[94]

superfamily of proteins, and are referred to as mini-ferritins (Fig. 8). These nanoparticles are ideal therapeutic carrier candidates similar to ferritin [105]. Dps are only found in prokaryotes, such as in bacteria and archaea [106]. At present, thousands of Dps-like proteins have been discovered and the majority (97%) are found to be in bacteria such as *E. coli*, and the remainder in archaea [106,107]. They are structurally conserved and differ only by the N-terminal and C-terminal regions of variable length which is responsible for the protein-DNA interactions [108]. Prokaryotes have existed for millennia, they have evolved to survive stressful conditions, and Dps is one key protein that aids in survival, protecting DNA from oxidative stress. This protective effect is achieved by the vital biochemical processes, such as iron sequestration,

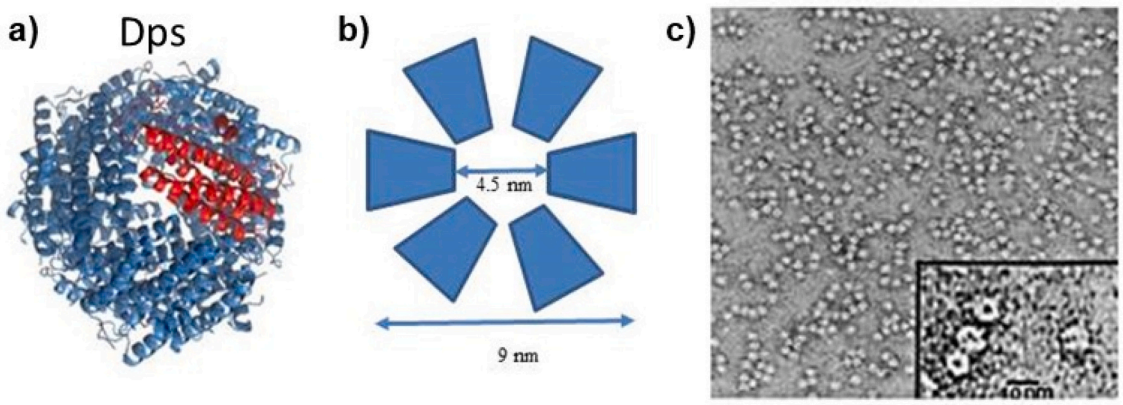

Fig. 8. (a) Canonical Dps forms a core of 12 subunits and displays 2-and 3-symmetry axes [38]. (b) Schematic representation of the structure of Dps which has an inner diameter of 4.5 nm and an outer diameter of 9 nm. (c) Electron microscopy images of negatively stained Dps [104].

ferroxidase activity, iron nucleation and DNA binding where all these activities occur in one Dps chain compared to ferritin which has two types - heavy and light chains [109]. However, Dps have many other functions too, such as conferring protection to cells from the toxic effects of heavy metals, heat, acid-base shock, UV and gamma radiation. Nevertheless, specific functions may depend on the species [110].

The crystal structure of Dps illustrates structural similarity to ferritin and the fundamental difference in terms of function between Dps and ferritins is that Dps binds to DNA. Dps is composed of 12 identical subunits that are assembled into a protein shell (ferritin has 24 subunits) and the molecular weight is approximately 19 kDa [104,111]. Dps depicts a protein fold identical to that of a ferritin monomer as it folds into a compact four-helix bundle suggesting a common evolutionary ancestor. The dodecamer consists of a hollow cavity similar to ferritin, where iron is stored. It is found that ~500 iron molecules can be stored per cage; the lower storage capacity compared to ferritin reflecting its smaller size [112]. It has an outer diameter of 9 nm and an inner diameter of 4.5 nm (Fig. 8) [108,113]. The scaffold is pH sensitive and disassociates at extreme pH levels such as pH 2.0, allowing cargo to be encapsulated, and reassembles into its normal structure at neutral pH. The external surface is negatively charged and contains pores that are formed at the junctions of the subunits. This allows cargo exchange in and out of the protein shell, hence offering potential benefits as a delivery carrier. The pores are found to be between 7 and 21 Å in diameter, depending upon its position [106]. Further, its biodegradability and biocompatibility becomes an added advantage when using it as a nano vehicle. Furthermore, due to its ultra-small structure, this nanoparticle could be used to target extremely narrow passages (areas inaccessible to ferritin molecules), and traverse biological barriers such as the BBB [114]. Although Dps are ideal nanoparticles, these proteins are less studied compared to ferritin [115]. With respect to use of Dps in the field of cancer research, very few studies have been reported to the best of our knowledge. Al-Ani et al., reported the use of Dps from *Listeria innocua,* which is a gram-positive bacterium (LiDps) in cancer cells. LiDps has been isolated by Bozzi in 1997, and it is able to protect DNA without binding to it. Al-Ani et al., developed a fusion protein combining *Gaussia princeps* luciferase and LiDps: (Gluc)-LiDps along with chemical conjugate; zinc (II)-protoporphyrin IX (ZnPP) targeting lysine residues on the fusion protein giving rise to Gluc-LiDps-ZnPP which is a photodynamic fusion protein. Reports have shown that photodynamic therapy is able to suppress tumour growth through ROS generation [116]. Bioluminescence Resonance Energy Transfer (BRET) between Gluc (470–490 nm) and ZnPP demonstrated the ability of the Gluc-LiDps-ZnPP conjugate to generate ROS (where ZnPP produces the ROS) resulting in cell death. This design might be very useful for deep tissue theranostic applications without the need for external energy. The activity of Gluc-LiDps-ZnPP, was tested against two breast cancer cell lines SKBR3, MDA-MB-231 and on MRC5 fibroblasts, and found significant growth inhibition in SKBR3 cells at concentrations >14.8 μg/ml in the presence of luciferin coelenterazine. In contrast, no growth inhibition was found in MDA-MB-231 and MRC5 cells; this may be a consequence of different uptake levels of photodynamic therapy by cells. Indeed, confocal microscopy revealed ~2-fold enhanced uptake of Gluc-LiDps-ZnPP by SKBR3 cells. The effect of Gluc-LiDps-ZnPP was tested in the presence of coelenterazine on SKBR3 cell migration where cell migration was also found to be reduced compared to control. Intriguingly, 2-fold higher intracellular ROS, was observed in SKBR3 cells treated with Gluc-LiDps-ZnPP in the presence of coelenterazine compared to the SKBR3 cells treated with each individual agent: Gluc-LiDps-ZnPP, Gluc-LiDps, and ZnPP. These results illustrate the potential of Dps to be used for cancer therapy where the fusion of Gluc and LiDps significantly enhanced the bioluminescence lifetime of luciferase Gluc [107]. Thus, Dps can be tailor-made to be used as a good delivery candidate for different kinds of therapeutics in cancer. Its exterior surface can be modified with various targeting ligands, and the hollow cavity can be used for the encapsulation of drugs or imaging agents. This work highlights the potential of small protein capsules for drug delivery. Comparative studies of the therapeutic efficiency of cancer agents encapsulated into different AFt capsules are needed. However, challenges associated with lack of systematic understanding of drug – capsule interior interaction and its effect on drug loading, retention and release need to be addressed.

6. Summary and perspective

Cancer is a leading cause of death worldwide and the modern therapeutic strategy for cancer is to target the tumour while minimising toxicity to healthy cells. Thus ferritin, which is the archetypal ferritin found in both prokaryotes and eukaryotes, has received intense attention in the recent past as a delivery vehicle that extends well beyond the scope of its primary role of iron storage. In this review we discuss a small selected set of examples where anticancer agents have been encapsulated in synthetic human AFt, horse spleen AFt and Dps. Our discussion supports that AFt is a promising nano platform for drug delivery, especially as it is biocompatible and fully biodegradable with built-in targeting, which could be exploited to deliver concentrated quantities of anticancer drug molecules to cancer cells and also to cancer stem cells which are known to repopulate tumours [117]. In current research, different types of small molecules and natural products are being encapsulated into AFt with a promising note in drug delivery and also in imaging, especially in the field of oncology. This is mainly because encapsulation within AFt improved water solubility, stability, bioavailability, cell absorption efficiency of the drug molecules. Further

AFt's exterior surface can be engineered to help enhance biocompatibility and tumour-targeting while the iron release pores can be used to fine tune release of molecular cargo. Nevertheless, modifications of the outer surface may alter the immunogenicity of the molecule upon entering the body. This may result in neutralising the therapeutic product and therefore reduce efficacy, or it can aggravate the situation by causing severe adverse consequences in the body, which warrants extensive research [118,119]. Furthermore, these excellent nano vehicles are able to passively penetrate the tumour tissues via the EPR effect, enhancing drug delivery to the tumour site; they also demonstrate the ability to traverse biological barriers such as the BBB via active targeting. Moreover, it was shown that horse spleen AFt which is more cost effective and readily available may have similar effects to human AFt as a delivery vehicle, compared to human AFt that requires complex synthetic biology for expression and purification. However, recombinant human AFt may have many advantages over horse spleen AFt since it is species-specific and it may show better biocompatibility and low immunogenicity in humans. Indeed, we cannot infer that different ferritin receptors function similarly. As a matter of fact, ferritins from different species may play contrasting roles beyond iron storage in various organisms [120]. Thus, more research is warranted to understand the mechanism and dynamics of AFt; such as, i) whether it is of human or horse origin, ii) possible chemotherapeutic agents that are encapsulated iii) drug retention and release iv) digestive stability of AFt v) encapsulation efficiency within AFt vi) cellular uptake efficiency vii) bio distribution, and clearance of this nanoparticle from the body to make this a promising vehicle for drug delivery and imaging in the field of oncology [23]. Furthermore, research is also necessary to address the possible immunogenicity that may arise in *in vivo* studies and ultimately in human clinical trials.

Similarly, Dps, a mini ferritin that belongs to the ferritin superfamily, demonstrates that it is a unique nano tool to be used as a drug delivery system. It is a protein that has evolved to protect bacteria from oxidative stress. However, there are many other activities among the members of the Dps family such as cell protection against multiple stresses, including metal stress, and heat and cold shock that require more detailed elucidation [106]. Further, the acquisition of nonspecific DNA-binding activity is beneficial when using Dps as it confers DNA-protective properties too. Nevertheless, only very limited investigations have been carried out compared to AFt, where we discussed an example illustrating Dps as a potential nano delivery candidate for anticancer agents. This suggests the need for more research with this unique nano molecule. However, these biocompatible, biodegradable biomolecules' benefits loom large in comparison to those of synthetic nano particles, thus, AFt and Dps should be explored extensively as putative theranostic delivery vehicles in future preclinical studies as well as in human clinical trials not least in the cancer arena.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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