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Converting Human Proteins into Precision Polymer Therapeutics

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Converting human proteins into precision polymer therapeutics

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Abstract: Cells as the smallest unit of life rely on precise macromolecules and programmable supramolecular interactions to accomplish the various vital functions. To translate such strategies to precisely control architectures and interactions into the synthetic world represents an exciting endeavor. Polymers with distinct structures, sequences and architectures are still challenging to achieve. However, in particular for biomedical applications, reproducible synthesis, narrow dispersities, tunable functionalities and additionally biocompatibility of the polymeric materials are crucial. Polymers derived from protein precursors provide many advantages of proteins such as precise monomer sequences and contour lengths, biodegradability and multiple functionalities, which can be synergistically combined with the valuable features of synthetic polymers e.g. stability, tunable solubility and molecular weights. The resulting polymeric biohybrid materials offer many applications ranging from drug delivery to biosensing and therapeutic hydrogels. This minireview summarizes the most recent advances in this field.

Keywords: Biopolymer, protein-polypeptide, drug delivery, NDs, hydrogel.

1. INTRODUCTION

In recent years, biomaterials research has focused on the design of both functional and biocompatible materials that accomplish multiple challenging tasks in biomedicine such as targeted drug delivery with intracellular specificity [1, 2], stimulus responsiveness in a cellular environment or inducible bioactivity [3]. In Nature, proteins realize most biological functions inside living cells. Their amino acid monomers are connected in distinct sequences so that precise 3D architectures are formed allowing controlled non-covalent interactions, which are critical for their rich biological activities. Protein-drug conjugates have successfully entered clinical phases [4] and a nanoparticle formulation of crosslinked human serum albumin (HSA) and the anti-tumor drug paclitaxel has already reached the market under the trade name Abraxane[5].

In order to increase the functional diversity nature offers, various drug transporters have been synthesized ranging from polymer drug conjugates over liposomes to silica nanoparticles (Figure 1) [6]. Polymers have been particularly attractive as versatile scaffolds for many biomedical applications e.g. drug or gene delivery as well as tissue engineering [7]. Programmed self-assembly into micelles and polymersomes has been explored to further expand their *in vivo* functions for guest encapsulation [8]. Even though synthetic macromolecules cannot be designed with similar levels of definition as natural biopolymers, important advances have been made to control their molecular weight and morphology [9, 10]. However, high biocompatibility, biodegradability and target specificity of polymer conjugates are still challenging to achieve.

An alternate strategy to accomplish polymeric materials with structural definition and rich functionalities focuses on exploiting proteins as polymeric precursors [11]. Proteins are ubiquitous in Nature and can be harvested from cells [12]. By combining both biological and chemical techniques, proteins and polypeptides of distinct monomer sequences, such as elastin-like and silk-like peptides, have been expressed and modified [13].

In this review, we will discuss the design and synthesis of protein-derived precision copolymers and elaborate structural modifications that have enabled their applications as drug transporters, nanoparticle coatings as well as modular hydrogels for various applications. We define precision polymers as polymers with a monodisperse backbone, distinct monomer sequences and functionalities at defined positions. Such precision polymers prepared from native proteins can be converted into versatile and multifunctional polymeric materials that offer great potential as tumor therapeutics, *in vivo* imaging or regenerative medicine [14, 15].

2. DESIGN OF COPOLYMERS FROM NATIVE PRECURSOR PROTEINS

It was proposed by Whitesides et al. that the chemical modification of a protein's side chains should facilitate narrowly dispersed polymers with distinct sequences of natural diversity [11]. The combination of a protein polypeptide backbone with grafted synthetic polymers as side chains yields semi-synthetic polymeric materials that provide many additive features of both protein and synthetic polymer constituents making them particularly attractive for biomedicine applications [16].

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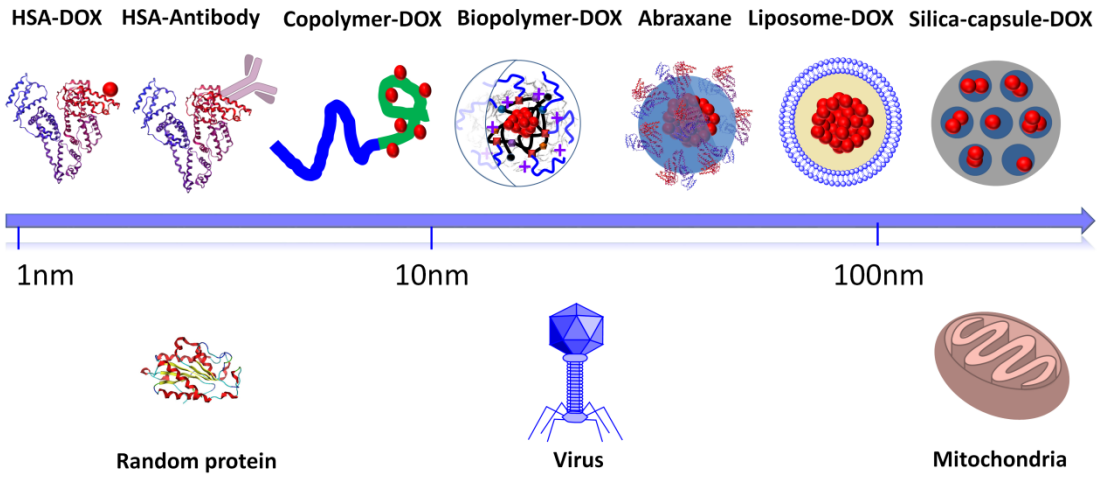

Figure 1. Drug transporters of different sizes are depicted in the upper part and comparison with biological architectures of different sizes in the lower part.

Due to the tightly folded polypeptide backbone of native proteins, many functional groups are not accessible for chemical modifications. Denaturing the 3D structure of proteins usually leads to destabilization and consequently aggregation of the polypeptide backbone [17, 18] thus preventing bioconjugation approaches. To overcome these challenges and further explore the potential of proteins as regenerative resource for polymeric materials, a comprehensive and versatile synthetic strategy was developed recently to access stable protein-based polypeptide copolymers [16, 19]. The preparation of such protein-derived polymers was based on the semi-synthetic "denaturation and stabilization" strategy. First, the protein precursor was dissolved in buffer with a chaotropic agent (such as urea or guanidinium hydrochloride) to induce denaturation and destabilization of the globular protein architecture followed by the addition of a reducing agent to cleave the disulfide bridges and improve the accessibility of reactive groups along the polypeptide chain. The resulting denatured polypeptides are usually insoluble over extended time periods due to the high number of hydrophobic amino acids of the protein backbone as well as oxidation and crosslinking of free thiol groups [19]. Therefore, in a second step, the reduced thiol groups of the cysteine residues were reacted with a solubilizing polymer to avoid refolding and precipitation of the unfolded polypeptide biopolymer. Polyethylene glycol (PEG) as the solubilizing side chains impart many attractive features such as low non-specific adsorption, potentially enhanced circulation in the blood stream as well as metabolic stabilization of the polypeptide chain (Figure 2).

Human serum albumin (HSA) [20], bovine serum albumin (BSA) [21], lysozyme [19], Ovomucoid [22] as well as trypsin inhibitor from *Glycine max* (Kunitz) [22] were converted into copolymers composed of varying molecular weights, chain lengths, ordered secondary structures [19]. The resulting PEG-copolymers are well soluble in water, stable during long term storage, non-cytotoxic and still degradable by proteases [23]. In addition, various reactive groups distributed along the polypeptide backbone are available to achieve multifaceted drug transporters [24]. Functionalization proceeded either before denaturation (*a priori*, e.g. cationization as shown in Figure 2) or after denaturation (*a posteriori*). *A priori* modification provides easier characterization since chemically modified globular proteins can be characterized by matric assisted laser desorption ionization time of flight (MALDI-ToF) mass spectrometry. In contrast, the denatured protein copolymers usually possess high molecular weights and substantial fragmentation during MALDI-ToF is often observed [25]. For instance, the precursor protein HSA was cationized by converting carboxylic acid side chains of aspartate and glutamate residues into primary amines and the degree of cationization was quantified by MALDI-ToF [26]. Copolymers derived from cationized HSA (cHSA) providing positively charged biopolymers with enhanced cellular uptake efficiencies as well as the high affinity to complex DNAs to facilitate gene delivery applications [27]. In comparison, *a posteriori* modification enables modifying most residues of the denatured polypeptide backbone including thiol groups of reduced cysteines, which can react efficiently and highly reproducibly. *Via* this approach, additional biorthogonal functionalities such as ethynyl groups to enhance the backbone hydrophobicity and accomplish ultra-small nanoreactors [28] as well as thioctic acid groups to stabilize

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and functionalize nanoparticles in aqueous solution were introduced [20].

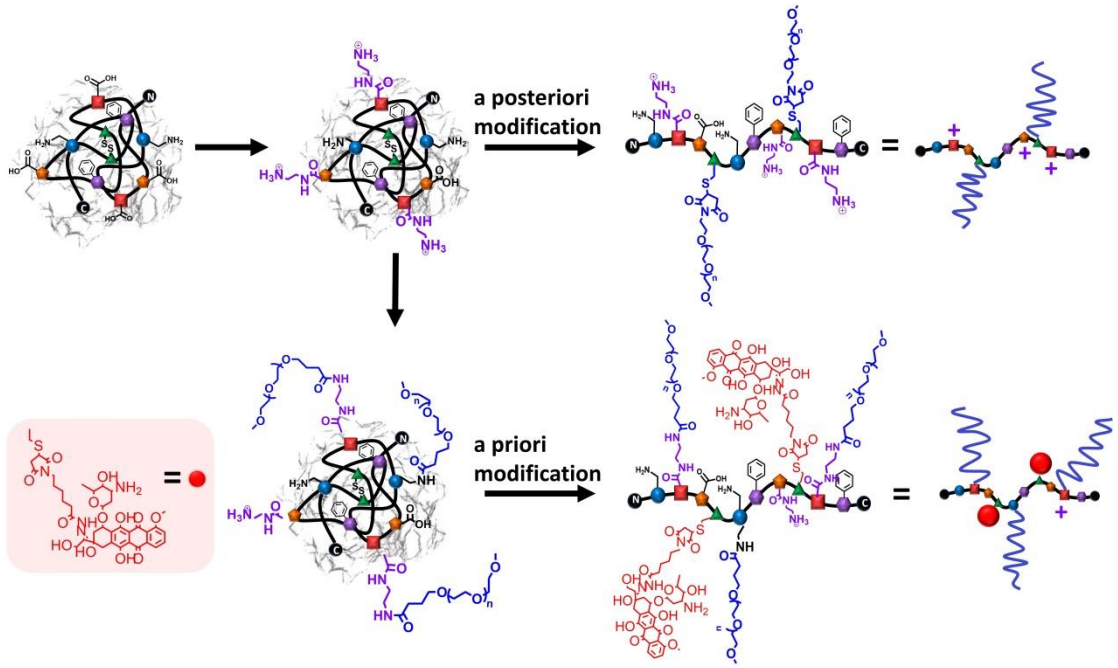

Figure 2. General scheme for functionalizing protein-derived copolymers. First, native HSA is denatured and cationized by converting negatively charged amino acid side chains into primary amine groups. A posteriori modification facilitates reacting PEG chains to the reduced cysteine groups yielding HSA-PEG or HSA-PEG-DOC copolymers

These highly versatile biopolymers represent unique polymeric materials featuring biocompatibility, biodegradability, multiple reactive groups along the backbone as well as the presence of ordered structural elements. Therefore, they are highly attractive for a broad range of applications such as bioimaging and drug delivery, which will be discussed in the following paragraphs. This "semi-synthetic" approach presented herein provides a valuable alternative to conventional polypeptide synthesis and expression approaches providing fast and efficient access to various polypeptides of natural diversity.

3. PROTEIN COPOLYMER DRUG TRANSPORTER

HSA-derived copolymers originate from the human protein HSA, one of the most abundant proteins in our blood plasma [16]. HSA has been extensively used as platform for drug delivery [4]. In particular anti-cancer drugs suffer from severe side effects due to their unspecific transport and interactions. To reduce side effects, efficient drug transporters are highly warranted. Drugs have been covalently as well as non-covalently bound to HSA [4]. Furthermore, HSA has been used in higher ordered structures like Abraxane [29], where several HSA molecules form a shell around a paclitaxel core. A major advantage of using HSA as transporter relates to its accumulation in tumor tissue also due to the enhanced permeability and retention (EPR) effect of macromolecules with molecular weights above 20kDa [30]. Carboxylic acid side chains of aspartate and glutamate residues were converted into positive charges by reaction with ethylene diamine to enhance cell membrane attachment and facilitate uptake via clathrin-mediated endocytosis. PEG side-chains were attached to available amino groups to contribute to reduced immunogenicity and antigenicity of proteins [31] and enhance stability and solubility. Doxorubicin (DOX) was chosen as representative anti-tumor drug molecule that could be loaded into the copolymers *via* two different strategies - hydrophobic encapsulation [24] and covalent conjugation *via* a cleavable hydrazone linker. Copolymers that form micelles in the presence of DOX molecules *via* hydrophobic interactions, revealed enhanced intracellular transport of DOX [24]. In order to control drug release and reduce non-specific drug leakage, about 27-28 DOX were covalently conjugated to the cysteine residues of the denatured cationized HSA-PEG conjugate *via* pH-sensitive hydrazone linker HSA-PEG-DOX to allow defined drug loading as well as controlled drug release in the more acidic targeted tumor tissue [16]. A two-step controlled drug released mechanism *via* (1) proteolysis of the albumin scaffold and (2) pH triggered hydrolysis of DOX from the HSA fragments inside the cell (Figure 3) was achieved. The anti-cancer activities of such albumin-drug conjugates were examined both *in vitro* and *in vivo* and potent cytotoxicity was found *in vitro* with HeLa cells as well as with different acute leukemia cell lines, with lowest IC_{50} of 1.9 nM for the MV4-11 cell line [16]. The efficacy of this delivery system was further confirmed by *in vivo* transplantation assays demonstrating significantly impairment of the engraftment potential of highly aggressive AML cell lines after 72 hr incubation [16].

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These preliminary *in vivo* transplantation studies further underlined the high efficiency of this macromolecular drug *versus* conventional application of DOX and suggested an attractive potential of this nanomedicinal formulation for targeting leukemia cells under *in vivo* conditions with a promising safety profile and minimized drug leakage. In addition, the large numbers of functional groups available on the protein backbone in principle allow introducing multiple functionalities into protein copolymers. For instance, a first protein based "theranostic" system was constructed by combining anti-cancer therapeutics and *in vivo* imaging groups such as nanoparticles as described in the next chapter.

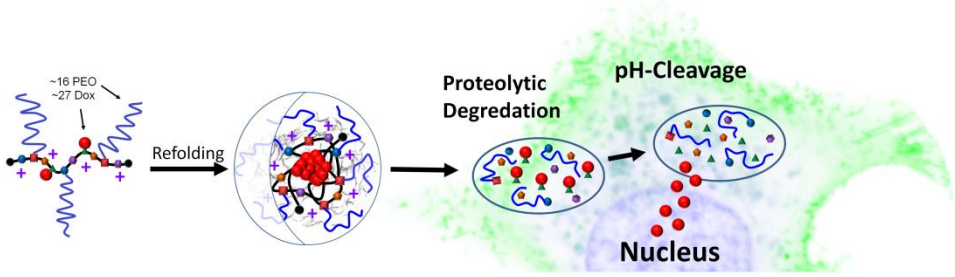

Figure 3. DOX loaded HSA-PEO-DOX for DOX delivery and two step drug release mechanism responding to enzymes and acidic pH.

4. PROTEIN COPOLYMER COATINGS FOR BIOIMAGING AND "THERANOSTICS"

In Nature, metal nanoparticles or nanoclusters are often stabilized by a protein shell, with Ferritin as prominent example [32]. Ferritin consists of 24 proteins subunits, which group around a central core of about 4000 iron atoms. Other examples include magnetotactic bacteria, which store iron particles in magnetosomes for magnetotaxis [33]. Inspired by these examples, nanoparticles were coated with functionalized protein-copolymers to impart a biocompatible hydrophilic stabilizing shell.

Semiconductor quantum dots (QDs) are commonly used as bioimaging agents providing high photo stability and brightness. However, their relatively large dimensions and cytotoxicity[34] can limit *in vivo* bio-labelling and bio-imaging applications and the preparation of QDs with excellent intracellular stability and solubility in various biological media is essential for such purposes. In this respect, QDs have been advanced further for bioimaging applications. Avian virus particles were labeled with QDs via biotinylation of the virus and addition of Streptavidin coated QDs [35]. The QDs allowed single particle tracking of the infection process over minutes, which facilitated visualizing the related receptor. Furthermore, "giant" QDs were achieved by ligand exchange with PEG, which were devoid of blinking [36]. An antibody labeled with such giant QDs facilitated the investigation of intercellular trafficking of membrane receptors. In another study, receptor oligomerization in living cells was studied with eight different QDs providing tailored emission features on a hyperspectral microscope [37]. In addition to single particle tracking, QDs provide many attractive features for the detection of tumors in tissue with high sensitivity. For example QDs were used in immunohistofluorescence imaging in a multiplexed assay with four different colors [38] and they revealed superior optical features over conventional fluorescent dyes. Fixed cancer cells in paraffin embedded tissue were identified via this method. Furthermore, QDs were used for multiplex diagnosis of colon cancer in mice [39]. Here, zwitterionic surface groups allowed to minimize unspecific adsorption, so that diagnosis could be made in vivo in mice and ex vivo in human colon tissue.

Until today, QDs have been successfully coated by different small molecules [40], polymers [41] as well as protein-derived copolymers [20]. Multivalent biopolymers with high affinity for the QD surface were achieved after attaching multiple thioctic acid anchor groups to the polypeptide backbone [20]. In this way, stable copolymer-coated QDs of about 30 nm dimensions were obtained [20] exhibiting exciting pH-dependent emission characteristics that were attributed to pH-dependent changes in the secondary structure of the protein-derived copolymer coatings [20]. As discussed above, QDs are also of great interest to transport drug molecules into the desired cells or tissue to visualize drug delivery processes. To address potential applications in gene delivery, QDs coated with polycationic protein copolymers were applied to condense DNA plasmids and photoresponsiveness was achieved upon DNA complex formation and release. These features offer mechanistic studies of non-viral gene delivery applying live-cell bioimaging techniques [16, 27].

Particles combining bioimaging capabilities and therapeutic drug molecules are generally referred to as theranostics [42]. Theranostics denote an emerging class of biomedical materials used as therapeutic and diagnostic agent in the same formulation. The idea behind this concept is to directly monitor drug trafficking to estimate the potential therapeutic success and side-effects at an early stage. Since every patient responds in an individual fashion to a respective

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drug dosage, theranostics might offer a more personalized treatment.

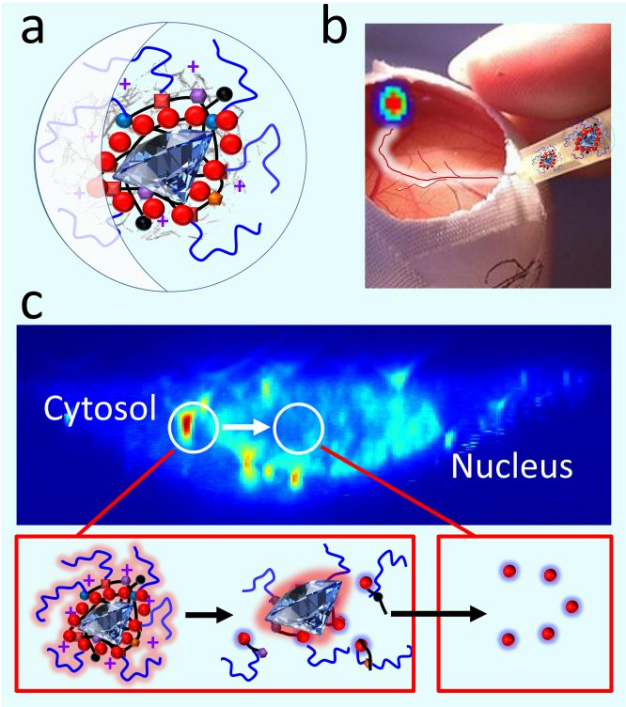

Figure 4. (a) Drug loaded biopolymer electrostatically coated fluorescent ND (DOX-fluorescent NDs) (b) DOX-fluorescent NDs for theranostic application in egg model (c) Fluorescent NDs monitored drug release in the cell cytosol.

In recent years, nanodiamonds (NDs) have received increasing interest because of their unique optical properties and presumably high biocompatibility due to the absence of any metals within the diamond lattice [43, 44]. NDs are nano-sized carbonaceous particles and substitution of single carbon with nitrogen atoms results in nitrogen vacancy centers exhibiting an extraordinary fluorescence without bleaching [45]. The combination of tunable colors, small sizes and low cytotoxicity makes NDs highly promising candidates as theranostics [46].

The application of NDs in life science is emerging and there have been already successful studies underlining their great potential in bioimaging and drug delivery. In the following, a few selected and recent findings have been summarized without the aim of giving an exhaustive overview of this evolving field. In vivo studies of NDs were reported with mouse lung cells labeled with NDs that were injected into mice to detect at main blood vessels [47]. In another study, NDs were coated with chelating ligands complexing gadolinium thus yielding an efficient MRI contrast agent [48]. Furthermore, NDs were evaluated as a novel drug delivery platform: Treatment with DOX loaded onto NDs revealed higher survival rates compared to free DOX [49]. Coating DOX loaded NDs with a functionalized PEG-lipid conjugate prevented tumor cells injected into mice from forming metastasized colonies in vivo. These results were compared to free DOX and DOX loaded NDs revealing improved features [50]. On the other hand, the DOX derivative daunorubicine was reversibly adsorbed non-covantently onto ND [51] and the resulting complexes were tested *in vitro* on a drug resistant leukemia cell line. Daunorubicine loaded particles were able to overcome the efflux mechanisms of a resistant cell line thus decreasing cell viablity. NDs were also exploited as gene delivery vectors. Micro RNA targeting proteins overexpressed in cancer cells, was loaded onto ND covered with protamine sulfate [52]. Upon delivery of the micro RNA to the cells, the expression of the specific proteins decreased as well as cell migration mobility, which could serve as first proof that NDs offer valuable features even for gene delivery applications. An interesting combination of the fluorescent ND delivery platform and light-induced toxicity was based on their combination with urchin-like gold/silver nanoparticles for photothermal therapy [53]. Uptake of such complexes into cancer cells was visualized via confocal microscopy and laser irradiation reduces cell viability significantly. Recently, a sophisticated theranostic ND conjugate was reported by connecting the epidermal growth factor receptor (EGFR) antibody and the drug Paclitaxel through a DNA linker onto the ND resulting multimodal ND drug delivery carriers [54] with enhanced uptake in cells expressing EGFR and significant cytotoxicity compared to the free drug.

The application of NDs in life sciences is often limited by aggregation of the raw particles and challenging functionalization. During production, NDs are usually obtained as negatively charged particles with many carboxylic acid groups at the surface [55]. Functionalization is therefore essential to suppress their strong tendency to form aggregates in water and high ionic strength buffers [55, 56]. Recently, their encapsulation into polymeric [57, 58] or inorganic shells [57, 59] was used to increase colloidal stability and introduce reactive functionalities for further conjugations (Table 1). Copolymers from cationonized HSA provide multiple positive charges that stabilized the ND surface via electrostatic interactions and PEG chains to efficiently prevent precipitation [20, 60]. Consequently, the rough and uneven ND surface was converted into a soft and more rounded biopolymer particle. To extend fluorescent NDs as platform for theranostics, DOX drug molecules were covalently attached to the copolymer coating through a pH sensitive hydrazone linker [60]. Such multifunctional NDs revealed a multilayer core-shell structure with a fluorescent ND in the center surrounded by the DOX loaded protein copolymer coating and finally a protective PEG hydration shell (Figure 4a). In comparison with other successful coating approaches, these particles exhibited high stabilities as well as many different reactive functionalities available at the surface (Table 1) [60]. Even loading of high numbers of hydrophobic DOX molecules had no impact on colloidal stability in the solid state as well as e.g. in high salinity buffer or even at extreme pH values. By high resolution confocal microscopy, the cellular uptake processes were monitored in real-time. Differences in fluorescence life times and photobleaching indicated efficient DOX release into the cytosol and trafficking into the nucleus, whereas fluorescent ND remained

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localized inside endosomes (Figure 4c). High *in vivo* efficacy was observed in a chicken chorioallantoic membrane model and dose dependent inhibition of tumor growth was demonstrated in human breast cancer xenografts [60]. Significantly increased antitumor efficacy compared to free DOX was obtained, proposing great potential of drug loaded NDs as metal-free theranostics providing optical diagnosis and chemotherapy with presumably improved biocompatibility.

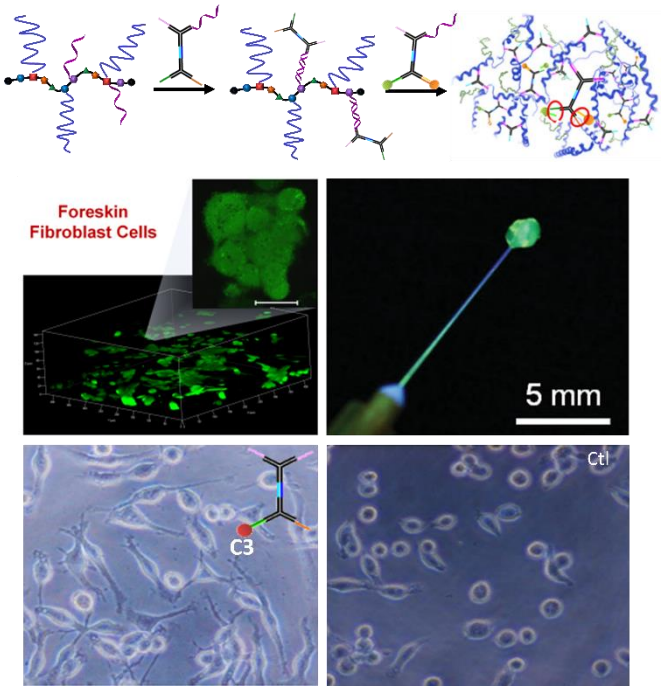
Table 1: Comparison of different nanodiamond coatings.

Coating method	Stability				Cytotoxicity	Chemical modification possibility	Ref
	pH range	Salt concentration	Physiological buffers	After loading hydrophobic drugs			
Silica coating	pH 5-8	Not reported	Not reported	Not reported	Not reported	Functionalization by established silica chemistry	[59]
Silica and PEG coating	pH 2-10	1M NaCl	PBS, tissue culture media	Not reported	Not reported	Can be modified via silica chemistry	[57]
PEG and copolymer coating	Not reported	150 mM	PBS	Not reported	Non-toxic until 50 µg/mL	No reactive groups for chemical modification available	[57]
Covalent PEG conjugates	Not reported	Not reported	PBS and DMEM + 10% FBS	Not reported	Non-toxic until 100 µg/mL	No reactive groups for chemical modification available	[58]
Hyperbranched polyglycerol covalent conjugation	Not reported	Not reported	PBS and DMEM	Aggregation found on TEM	Non-toxic until 400 µg/mL	Can be modified at the hydroxyl end groups.	[61], [62]
Albumin biopolymer coating	pH 2-8	1 M	PBS, HEPES and DMEM + 20% FBS	Stable in PBS and cell culture medium and even 1 M NaCl	Non-toxic until 3 mg/mL	Large numbers of different functional groups available	[20], [60]

5 HYBRID HYDROGELS

Hydrogels consist of networks of physically or chemically connected hydrophilic molecules, which swell in water. Due to their high water contents, hydrogels represent an emerging class of materials in tissue engineering and drug delivery [63-65]. For biomedical applications, hydrogels need to be biocompatible and biodegradable as well as of tunable stiffness and they should allow straight forward introduction of additional functionalities. Biocompatible and biodegradable copolymers from protein precursors offer many desired features and hydrogel formation can be achieved by imparting suitable crosslinkers [66]. Oligonucleotides were introduced as "natural" supramolecular gelators that do not require the presence of catalysts or toxic reagents. Y-shaped single stranded oligonucleotide cross-linkers (ssDNA) were designed that allowed connecting different polypeptide backbones containing complementary ssDNA sequences and gelation occurred immediately after combination of the polypeptide copolymer backbone and the DNA crosslinkers. Stiffness of the hybrid hydrogel was tunable in the range of ~1 to ~4200 Pa, which is notably higher than hydrogels made up entirely of peptides [67] or DNA [68] resulting most likely from the high content of helical structures of the HSA-derived polypeptide precursors. The gelation process based on DNA hybridization proceeds in the absence of toxic compounds or catalysts thus providing a powerful approach even for the encapsulation of living cells. Human lung adenocarcinoma cell line A549 and human foreskin fibroblast were used in 3D cell culture [66] demonstrating excellent biocompatibility and highly designable properties of these hybrid hydrogels.

Figure 5. Hydrogel formation with protein-polymer conjugates carrying PEG



chains (blue lines) and single stranded DNA sequences (purple lines) attached to the polypeptide backbone of denatured HSA. Hydrogel formation proceeded after applying DNA crosslinkers binding to DNA of the polypeptide backbone and carrying the desired functionalities (green and orange circles). Lower row from left to right, 3D culture of foreskin fibroblast cells, injectability of hydrogel components, cell morphology change after release of C3 from hydrogel.

Functional hybrid hydrogels were achieved by applying DNA crosslinkers containing proteins (shown in Figure 5 as green and yellow circles) that were incorporated into the

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hydrogel by DNA hybridization. X-shaped Origami crosslinker containing four arms in total, as depicted in Figure 5, provide two arms, which were functionalized with up to two different functionalities, e.g. dyes or fluorescent proteins thus enabling simultaneous crosslinking and functionalization within one reaction step. Such hybrid hydrogels consisting of a polypeptide backbone and oligonucleotide crosslinkers were biodegradable by proteases as well as nucleases to release the respective cargos upon an external enzyme stimulus. The potential of such hybrid hydrogels for in vivo applications is currently being investigated.

7 SUMMARY

Copolymers derived from protein precursors represent a new and versatile platform in biomedical research. The major advantages of these copolymers include their facile production from abundant, relatively inexpensive starting materials, their low cytotoxicity, biodegradability and defined backbone length providing many different functionalities at defined positions suitable for bioconjugation reactions. These functionalities can be chemically modified following different approaches to tune the functional properties of the protein copolymers. For example cationization resulted in increased cellular uptake, tight interactions with ND surfaces, whereas the attachment of thioctic acid allowed coating to QDs. This modular approach enabled the covalent attachment drug molecules even with cleavable linkers. In addition, the decoration of copolymers with oligonucleotides facilitated the *in situ* formation of hybrid hydrogels for the encapsulation of living cells. The versatility and functionalizability of these biopolymers offers great opportunities for many different biomedical applications ranging from drug delivery, hydrogel formation to bioimaging.

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