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Albumin-bound nanodiscs as delivery vehicle candidates: Development and characterization

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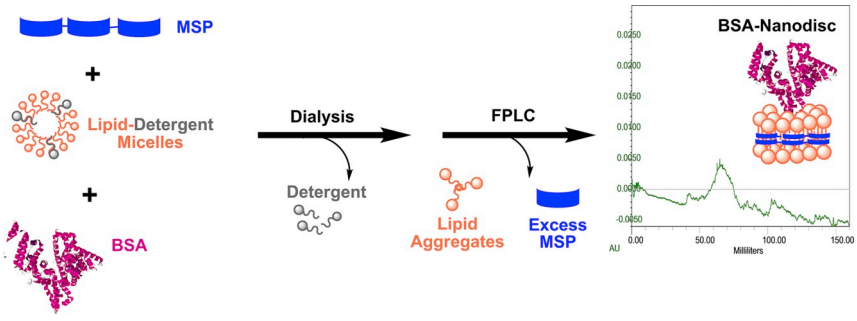
HIGHLIGHTS

- Synthetic HDL-like phospholipid nanodiscs are constructed by POPC, MSP, albumin with/without FITC as a vehicle candidate.
- Impact of BSA adsorption onto nanodiscs surfaces has been studied.
- Influence of fluorescent labeling of albumin on several properties of albumin-bound nanodiscs has been investigated.
- This work presents for the first time an attempt to modify nanodiscs with albumin as an effective drug carrier candidate.

GRAPHICAL ABSTRACT



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ABSTRACT

Development of synthetic bioarchitectures to improve our understanding of biological systems and produce biosynthetic models with new functions has attracted substantial interest. Synthetic HDL-like phospholipid nanodiscs are a relatively new model of nanoparticles that present a promising carrier for drug delivery and membrane protein investigations. Nanodiscs are soluble nanoscale phospholipid bilayers that are produced based on the self-assembly of phospholipids, membrane scaffold proteins (MSP) and an embedded peptide/protein of interest. To determine the effect of conjugating a protein with a probe, the model protein bovine serum albumin (BSA) with or without FITC conjugation was attached onto 100% 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-choline (POPC) nanodiscs. The generated discs were analyzed by Fast Protein Liquid Chromatography (FPLC), dynamic light scattering (DLS), and UV-VIS spectroscopy. Empty, BSA- and FITC-BSA-Nanodiscs exhibited different size, charge and elution characteristics as well as different release profiles. Thus, conjugation of proteins to be adsorbed onto nanodiscs surfaces with fluorophores can affect the physical and release properties of nanodiscs, thereby potentially impacting their biophysical, delivery and imaging applications.

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1. Introduction

Progress in nanotechnology is revolutionizing the encapsulation of recombinant proteins, drug delivery and the productivity of pharmaceutical R&D. Nanomedicines allow the use of natural or biomimetic materials like lipids as carriers to deliver hydrophobic as well as hydrophilic molecules, preferentially to target disease sites to enhance bioavailability [1–5]. Nanomedicines can improve pharmacokinetic and pharmacodynamic properties, and hence, the therapeutic potential of a drug molecule. Nanosystems such as micelles, liposomes, nanodiscs, dendrimers, nanocrystals and silica nanoparticles are promising platforms to solubilize or immobilize membrane proteins and provide effective delivery [6–12]. Although liposome-based drug delivery systems have already been marketed for a variety of drugs, liposomes have some drawbacks [7]. The limitations of conventional liposome-based drug delivery systems include short circulating half-lives, limited capacity for drug encapsulation, and instability in biological fluids [10,13,14]. Nanodiscs have been introduced as an alternative approach for the reconstitution of membrane proteins in a biomimetic lipid membrane and as drug carriers that can overcome some of the limitations of liposomes [15,16].

Nanodiscs are novel synthetic HDL-like phospholipid particles that were first developed by Sligar et al. in the early 2000s. This model membrane system is 10–12 nm in diameter with a thickness of 4.6–5.6 nm depending on the lipid used and corresponds to the thickness of a biological membrane [17]. These discoidal particles consist of a phospholipid bilayer surrounded by two membrane scaffold protein (MSP) molecules [6,18–20]. MSPs are amphipathic peptides derived from apoprotein A-I, which is an important protein in the human bloodstream that forms high-density lipoprotein (HDL). In a nanodisc system, the MSP coat provides enhanced stability and a homogeneous particle size distribution by maintaining bilayers in well-defined nanometer size. Changing the diameter of the protein coat precisely controls the diameter of the lipid bilayer [16]. Nanodiscs are generated based on the self-assembly of phospholipids, MSP and an embedded peptide/protein of interest, upon detergent removal. Since the nanodisc structure closely mimics the native physiological arrangement, they constitute an important tool for studying the structure and function of proteins. In contrast to liposomes and micelles, nanodiscs better reflect the complex phase transition behavior of biological membranes. They are relatively more stable over time than conventional model membranes due to the protein coat [21,22], allow access to the embedded protein from both sides of the disc, provide a native-like environment to incorporated proteins and maintain correct folding [19,23], and increase the duration of drug circulation in the bloodstream [24]. Unlike other lipid nanoparticles, nanodiscs contain an additional component, MSPs, which significantly affect the structure, function and physical properties of nanodiscs [25–28]. Nanodiscs have smaller number or fewer types of lipid molecules, but lipid shape can be an important determinant for the function of the incorporated protein. Many nanodiscs are assembled using a single type of lipid, *e.g.*, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), or a mixture of lipids such as phosphatidyl-ethanolamine (PE) and phosphatidylserine (PS). The reconstitution of peptides, proteins, or therapeutic agents in nanodiscs provides promising functionality for several substances such as cytochrome P450 [29], bacteriorhodopsin (bR) [23] and kappa opioid receptor (KOR) [30]. However, there are insufficient studies concerning stability, charge interactions and forces acting between MSPs and other molecules in nanodiscs.

The development of nanocarrier system plays a crucial role in drug delivery to treat life-threatening diseases. Many natural biomolecules such as peptides and proteins are exploited in drug formulation due to their safety, biocompatibility and biodegradability. Moreover, they can be used as agents that improve the stability and efficacy of the delivery systems. Among biomacromolecule proteins, albumin is a significant plasmatic carrier that is commercially available with wide uses in nanocarriers development and drug delivery. Albumin is a small globular monomeric and heart-shaped protein. In the circulating system, albumin is the main soluble protein that is involved in nutrient transportation to the cells and maintenance of osmotic pressure [31]. Albumin-based nanoparticles are drug delivery platforms that exploit the unique properties of albumin for binding and transportation to carry hydrophobic drugs and enhance tumor penetration [32]. In the current study, the model protein bovine serum albumin (BSA) was assembled with nanodiscs. BSA is routinely used as a model protein as an alternative to human serum albumin (HSB) due to their structural homology [33]. BSA is a negatively charged protein at physiological pH (isoelectric point is 4.7) and consists of 583 amino acids with a molecular weight of approximately 66 kDa. Furthermore, BSA is widely used and extensively studied protein because of its availability, stability, lack of interference with biochemical reactions, unique ligand-binding properties, low cost and ease of purification from bovine blood [34]. The bioconjugation of two molecules forms a new material with characteristics resulting from new or the combined properties of the individual components. BSA is commonly conjugated with the fluorescent probe fluorescein isothiocyanate (FITC), also a negatively charged molecule with a molecular weight of approximately 390 Da. Modifying any protein with a fluorescent label makes the protein visible and easily detectable through different techniques, such as imaging and fluorescence spectroscopy.

Due to the established value of albumin in drug delivery, this work aimed to develop an albumin-bound nanodiscs (Fig. 1) and to determine the influence of the binding of BSA and FITC-conjugated BSA on the physical properties of nanodiscs including particle size, zeta potential, and stability. Charge measurements were used to determine the degree of repulsion between nanodisc components, including lipids, MSP, BSA and FITC molecules. Empty, BSA- and FITC-BSA-nanodiscs differed in their zeta potentials and physical properties.

2. Materials and methods

2.1. Synthesis of nanodiscs

A stock solution of 20 mg/mL of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) lipid (Avanti Polar Lipids) was prepared by dissolving the lipid in chloroform, stored at −20 °C, and used to prepare different types of nanodiscs. The required amount of POPC lipid was dried under a gentle stream of nitrogen and desiccated overnight to completely remove the organic solvent. The dried film was then resuspended in 500 µl of 20 mM Tris-HCl (pH 7.4) buffer containing 100 mM sodium deoxycholic acid and incubated at 37 °C for 20 min. Two milligrams of MSP1D1-His lyophilized protein (Cube Biotech, Germany) were resuspended in 20 mM Tris-HCl (pH 7.4) buffer and used directly. To generate empty nanodiscs, MSP and POPC were mixed at the molar ratio of 1:65 MSP:lipids and incubated at 4 °C with gentle shaking. After 2 h, the nanodisc mixture was added to a dialysis tube (ZelluTrans, MW 3500 Da) and dialyzed against 20 mM Tris-HCl (pH 7.4) buffer for 2 days at 4 °C to remove the detergent. The dialysis buffer was changed 4 times during this period to ensure near complete removal of the detergent. After detergent removal, samples were analyzed for absorbance (280 nm) and conductivity and purified by Fast Protein Liquid Chromatography (FPLC). Samples were applied to a Superdex 200-HR column (XK-16 column, 16 × 600 mm, GE Healthcare) at a flow rate of 0.5 ml/min. Collected fractions were concentrated (Amicon® Ultra 15 mL centrifugal filter devices, Merck) and analyzed by SDS-PAGE. To generate BSA- and FITC-BSA-nanodiscs, the same protocol used for empty nanodiscs was followed and BSA (Sigma Aldrich, A7906) or FITC-BSA (Sigma Aldrich, A9771) molecules were added to the MSP solution at a molar ratio of 1:20 BSA:MSP.

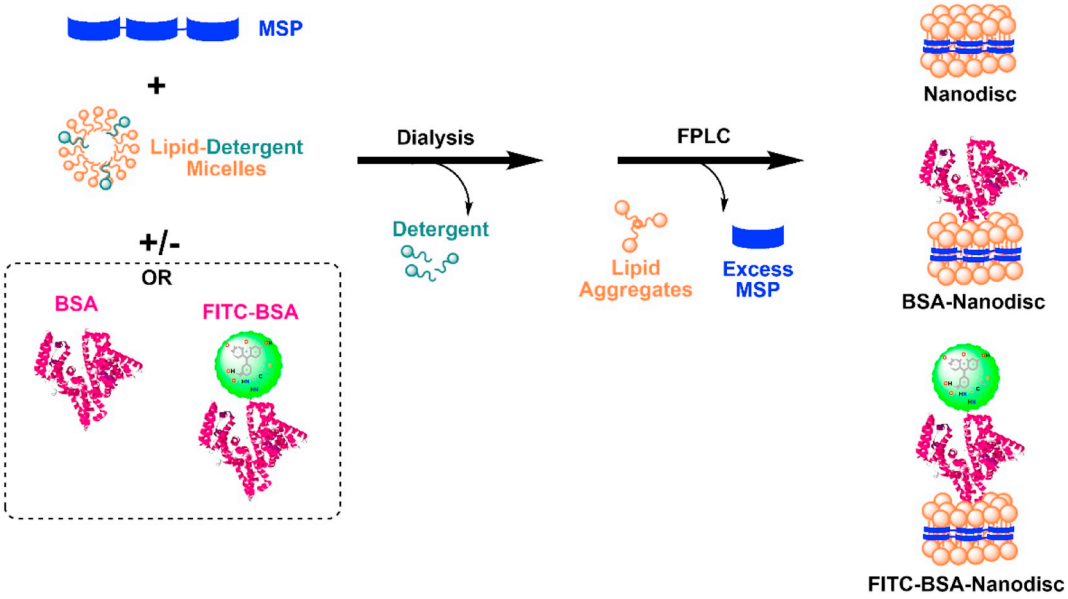

Fig. 1. Assembly of empty-, BSA-, and FITC-BSA-nanodiscs. Nanodisc self-assembly showing POPC, MSP1D1-His, BSA, and FITC-BSA. Disc generation is initiated by detergent removal through dialysis, followed by a purification step using FPLC.

2.2. Measurement of hydrodynamic size and zeta potential

Hydrodynamic size, polydispersity index (PDI) and zeta potential were investigated by dynamic light scattering (DLS) using a Zeta Sizer Nano-ZS (Malvern Instrument). The nanodisc samples were diluted with 10 mM Tris-HCl (pH 7.4) prior to analysis at 25 °C. One-way ANOVA with Kruskal-Wallis test was performed to compare the level of difference between empty, BSA and FITC-BSA nanodiscs. Differences were considered to be significant at $P < .05$.

2.3. SDS-PAGE

All nanodisc samples were diluted in NuPage LDS buffer with reducing agent (Thermo Fisher) and heated at 70 °C for 10 min before gel electrophoresis. Afterward, samples were loaded onto a 10% PAGE gel (Novex) and separation was done at 170 V for ~60 min. Subsequently, the gel was stained using ready-to-use Coomassie stain (Invitrogen) for one hour at room temperature with shaking, followed by washing with distilled water (1 − 3h) until clear bands were observed.

2.4. In vitro release studies

Ultraviolet visible (UV) spectroscopy was used to monitor the stability of the generated nanodiscs, in terms of protein release. The release behavior of the proteins (MSP alone or with albumin) was determined by dialysis against PBS (pH 7.4) at 37 °C. A nanodisc solution was sealed in a micro-dialysis device (MW 100 kDa, Micro Float-A-Lyzer, Spectrum labs) and immersed in 10 mL PBS with continuous agitation. At predetermined time intervals, 100 µL of PBS was removed and replaced with the same amount of fresh PBS. The amount of released protein was determined by UV spectrophotometry using the BCA protein assay according to manufacturer's protocol.

3. Results

3.1. Formation and purification of nanodiscs

FPLC was used to monitor the formation of nanodiscs and the attachment of BSA or FITC-BSA to the nanodiscs, as well as to purify the nanodiscs and remove any excess lipids or proteins from nanodisc solutions (Fig. 2). As expected, free albumin eluted last. A chromatogram of empty nanodiscs shows that the majority of generated particles were eluted as a sharp monodisperse peak, whereas BSA, and FITC-BSA-nanodiscs display broad elution peaks. FPLC analysis shows the main peaks eluting at approximately 38 mL for empty and BSA-nanodiscs, while FITC-BSA-nanodiscs show the peak maximum at 39 mL. It is obvious that the attachment of protein molecules to nanodiscs affects elution characteristics, such as the peak shape and elution time. The prolonged elution of FITC-BSA-nanodiscs may be attributed to interaction of FITC-BSA with the resin and transient binding to the Superdex matrix. FITC-BSA-nanodiscs displayed prolonged elution times compared to empty and BSA-nanodiscs. The shift in elution volume of approximately 1 mL may correspond to the presence of FITC and may indicate more heterogeneous species. Hence, it is obvious that attachment of FITC clearly affects the nanodisc properties.

3.2. SDS-PAGE analysis

SDS-PAGE was used to confirm the binding of BSA or FITC-BSA with the nanodiscs under reducing conditions (Fig. 3). Samples were loaded onto the gel before and after FPLC. However, FPLC eluted samples were concentrated before loading, and therefore intense bands appeared due to the higher protein content of these samples. In the case of empty nanodiscs, the results show a highly pure system with one distinct band for MSP (~23 kDa), which is slightly smaller than the calculated molecular weight of MSP (25 kDa). Free BSA and FITC-BSA proteins were loaded onto the gel to compare against the proteins attached onto the nanodiscs. BSA-nanodiscs show two distinct bands: the MSP, running at ~23 kDa, and the BSA, running at ~66 kDa, which is in good agreement with free protein. Similar to BSA-nanodiscs, FITC-BSA- nanodiscs show a distinct band for the MSP and display multiple bands that may be attributed to FITC that has detached from albumin.

3.3. Estimation of the size of nanodiscs formed

Table 1 shows the hydrodynamic size of empty-, BSA-, and FITC-BSA-nanodiscs as measured by DLS. The average hydrodynamic particle size of empty POPC nanodiscs is 9.53 ± 0.48 nm in diameter, which is close to the previously reported size [16]. The attachment of albumin to nanodiscs increased the size of the nanodiscs slightly compared to empty nanodiscs (12.28 ± 1.84 and 13.41 ± 1.90 nm for BSA- and FITC-BSA-bound nanodiscs, respectively) (Fig. 4).

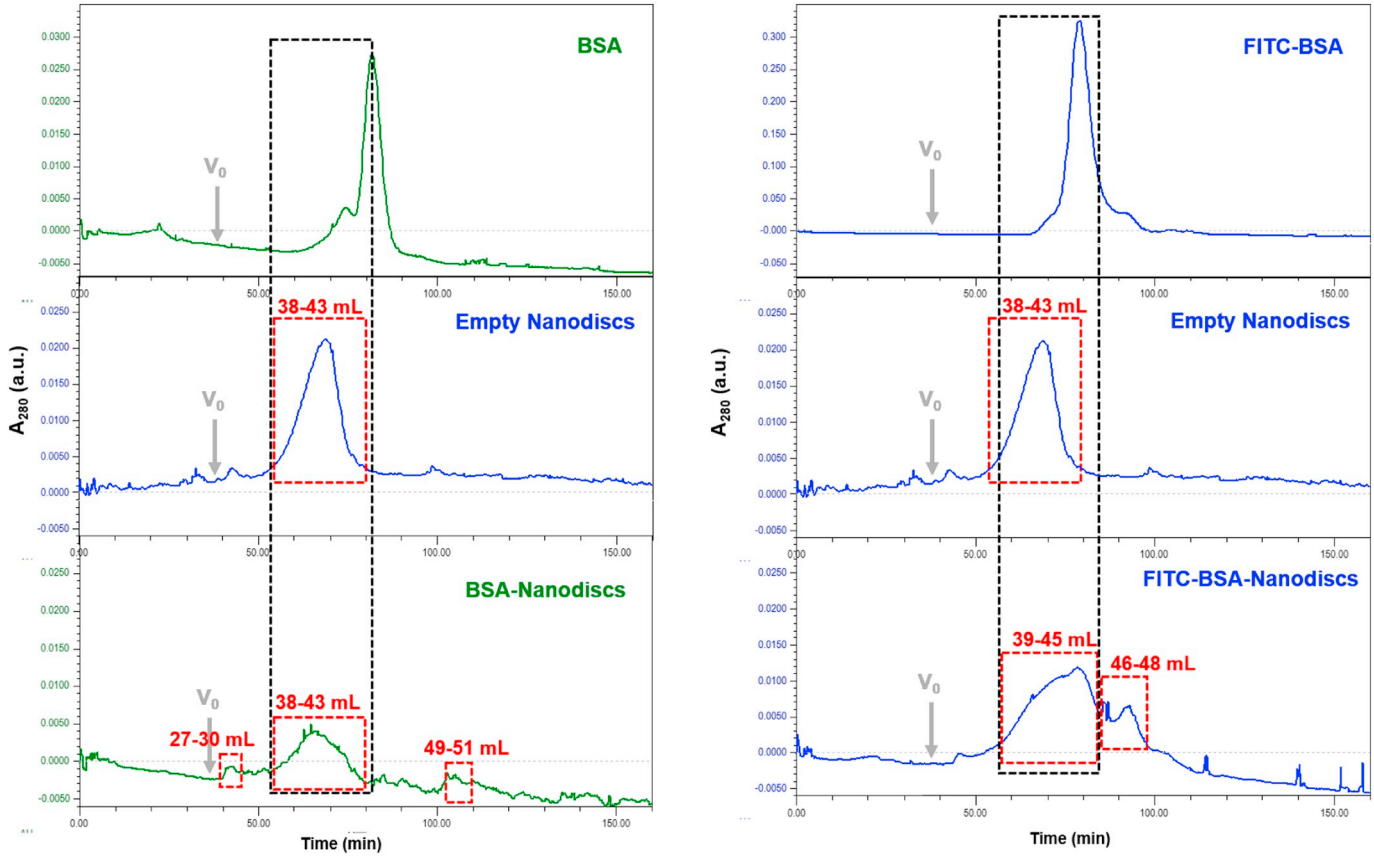

Fig. 2. Left: FPLC chromatograms of free BSA, empty nanodiscs, and BSA-nanodiscs. Right: FPLC chromatograms of free FITC-BSA, empty nanodiscs, and FITC-BSA-nanodiscs. Elution profiles of main and minor peaks correspond to eluted nanodiscs and lipid aggregates, respectively. Red dashed rectangles show the collected fractions. Black dashed rectangles show the integrated area between free (FITC)-BSA, empty nanodisc, and bound nanodiscs. FPLC elution of the generated nanodiscs was performed on a Superdex 200-HR column at a flow rate of 0.5 mL/min with UV detection set at 280 nm. The mobile phase was 20 mM Tris-HCl (pH 7.4) buffer. V_0: void volume. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Characterization of the zeta potential of nanodiscs

Nanodisc charge was quantified by measuring the zeta-potential, which measures the electrophoretic mobility of the discs in an electrical field. As shown in Table 1, changes in zeta-potential and electrophoretic mobility values for empty-, BSA-, and FITC-BSA-bound nanodiscs indicate changes in surface charge that may reflect the successful adsorption of albumin molecules. In the absence of BSA protein, the zeta potential of empty POPC nanodiscs was -24.03 ± 1.86 mV, whereas after the attachment BSA and FITC-BSA, the nanodiscs became less negative by approximately 19.3% and 13.3%, respectively (-19.4 ± 1.18 and -20.83 ± 2.47 mV, respectively), due to the

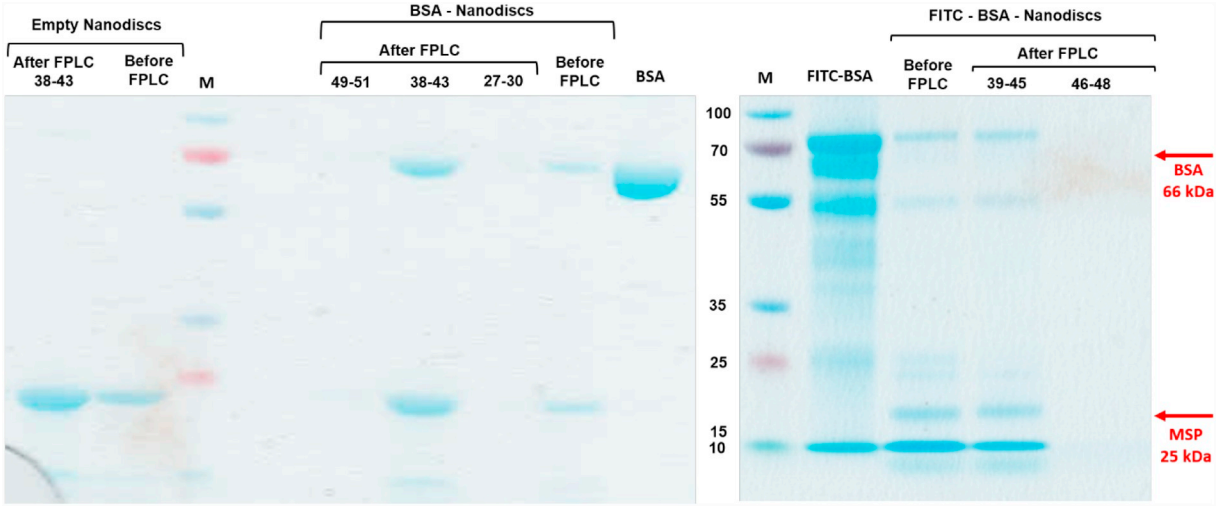

Fig. 3. SDS-PAGE analysis of free BSA, free FITC-BSA, and eluted minor and major peaks from FPLC for empty-, BSA- and FITC-BSA-nanodiscs in reducing conditions and compared with protein molecular mass marker (M). The eluted fractions 38–43 mL and 39–45 mL indicated that BSA and FITC-BSA nanodiscs eluted at those fractions, respectively, while eluted fractions 27–30 and 49–51 mL for BSA nanodiscs and 46–48 mL FITC-BSA nanodiscs may be attributed to lipid aggregation.

Table 1
Physical parameters of empty-, BSA- and FITC-BSA-bound nanodiscs derived from DLS measurements ($n = 3$). Values are the mean ± SD; Comparisons of the *Z*-average size hydrodynamic diameter and zeta potential were tested by the Kruskal-Wallis ($P < .05$); n.s., not significant.

Nanodiscs	Size	Volume	PDI	Zeta potential	Electrophoretic mobility
	(d.nm)	(%)		(mV)	(μmcm/*Vs*)
Empty	9.53 ± 0.48	97.4	0.44 ± 0.21	−24.03 ± 1.86	−1.93 ± 0.12
BSA	12.28 ± 1.84 n.s.	98.4	0.48 ± 0.09	−19.4 ± 1.18 n.s.	−1.55 ± 0.09
FITC-BSA	13.41 ± 1.90 n.s.	97.8	0.39 ± 0.09	−20.83 ± 2.47 n.s.	−1.29 ± 0.07

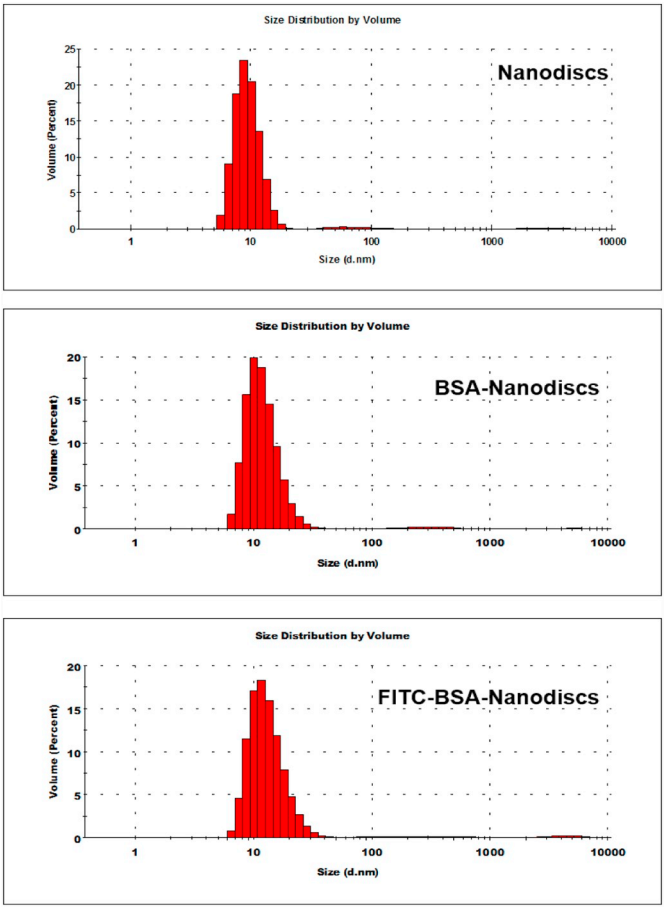

Fig. 4. Hydrodynamic size distributions based on DLS measurements and volume percent for different nanodiscs.

negatively charged molecules (MSP, BSA, and FITC) and surface modifications (Fig. 5).

3.5. Nanodisc stability analysis

The BCA protein assay was used to quantify proteins released form the nanodiscs as an indicator of nanodisc stability. The cumulative release of MSPs and BSA/FITC-BSA proteins from the nanodiscs was analyzed *in vitro* over an experimental period of 4 days in PBS solution. As shown in Fig. 6, empty nanodiscs did not exhibit an initial bulk release of MSPs, which indicates the stability of the generated nanodiscs and the slow degradation of the MSP belt that surrounds the lipid molecules. BSA-nanodiscs exhibited faster release kinetics than other nanodiscs. The initial burst release of the protein reached nearly 50% of the total protein released within the first six hours which may attributed to albumin release more than MSP. In contrast, in the case of FITC-BSA-nanodiscs, released proteins reached approximately 50% of the total protein released after 4 days.

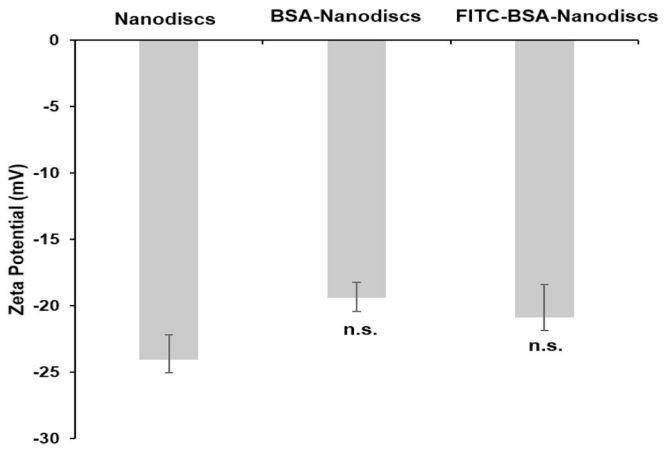

Fig. 5. The zeta potential of empty-, BSA- and FITC-BSA-nanodiscs. Values are mean ± SD ($n = 3$); n.s., no significant difference when compared to empty nanodiscs.

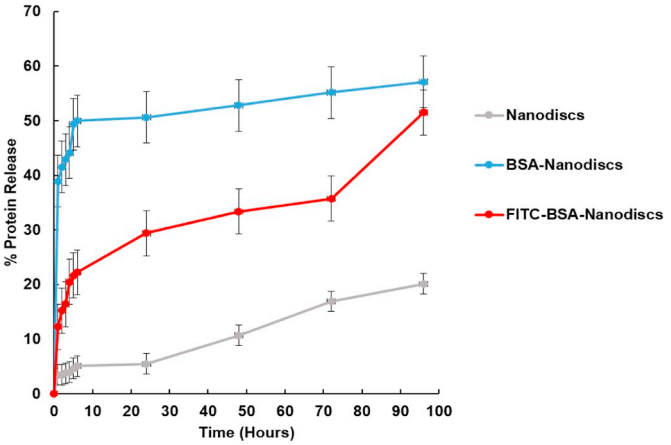

Fig. 6. Cumulative release of MSP and albumin proteins form generated nanodiscs during 4 days at 37 °C ($n = 3$).

4. Discussion

Nanodiscs are attracting attention as a promising membrane mimetic for the reconstitution of membrane proteins and as effective vehicles for drug delivery. The careful choice of nanodisc components (lipids and MSPs) plays a significant role in the functionality of these particles. In this study, 100% POPC and MSP1D1-His were chosen as they have been extensively used to generate nanodiscs. There are four main parameters that dominate formation of nanodiscs: (i) ratio of lipid:MSP, (ii) choice of detergent, (iii) temperature, and (iv) ratio of MSP:other protein [19,35,36]. POPC is a zwitterionic, cylindrical phospholipid that adopts a lamellar phase [37]. Generally, the lipid:MSP ratio depends on the type of lipid and therefore, the ratio should be optimized to generate nanodiscs. The lipid concentration should be higher than MSP molecules to allow self-assembly and the

formation of nanodiscs. However, high lipid contents lead to aggregation while low lipid contents lead to poor nanodisc formation [38–40]. At a high ratio of target protein to MSP, multiple protein molecules may be incorporated into a single nanodisc, which may lead to non-specific interactions between proteins incorporated into the same disc. In contrast, at a low ratio of target protein to MSP, an excess of empty nanodiscs leads to diluted target protein sample [41–43]. Furthermore, detergent concentration plays important role as it needs to be twice the concentration of lipids to initiate the self-assembly process and nanodisc formation. According to Sligar's protocol, the concentration of cholate should be in the range of 12–40 mM. In this work, for POPC nanodiscs, the optimal molar ratio has been established previously by Sligar and colleagues as 65:1 (POPC:MSP) and extra concentration of cholate (100 mM) was added to end up with 20 mM as a final concentration to allow proper self-assembly process [19,38,39]. Previous studies have shown that POPC nanodiscs exhibit low salt dependency and are stable in different salt types and concentrations at a pH ranging from 7.0 to 8.5 and a temperature ranging from 20 to 35 °C. In this study, nanodiscs were prepared using the detergent dialysis method according to a procedure described by Matz and Jonas [41]. The formation of POPC nanodiscs is usually conducted at 4 °C since the main transition temperature of POPC is 2 °C [42]. In this work, the MSP:BSA or MSP:FITC-BSA ratios used were 20:1. However, FPLC analysis showed that FITC-BSA-nanodiscs exhibit higher, wider peaks than BSA-nanodiscs, which may be attributed to the presence of the FITC isomer and the arrangement and behavior of FITC-BSA molecules with the nanodiscs.

As seen in Fig. 3, BSA-nanodiscs exhibit two distinct bands correspond to MSP and BSA, while FITC-BSA system displays multiple bands that might be attributed to detached FITC from albumin. FITC is coupled to albumin through the amino groups of lysines in albumin, and the degree of substitution is 7 to 12 mol of FITC per mole of albumin [43]. BSA tends to form inter-protein multimers *via* disulfide bonds [44]. Presence of the reducing agent (pH 8.4) improves formation and reshuffling of disulfide bonds through cysteine oxidation and thiol interchange reactions, respectively [45]. However, all bands attributable to FITC-BSA-bound nanodiscs correspond to free FITC-BSA and MSPs. SDS-PAGE analysis of the minor FPLC peaks showed no proteins bands, indicating that these peaks are attributable to lipid aggregation resulting from excess POPC molecules.

The sizes of nanodiscs are dominated by two factors: the length of the MSP and the number of lipids per bilayer area. Typically, MSP nanodiscs have a bilayer area of nearly 4400 $Å^2$ and approximately 126 total lipids (each monolayer has approximately 63) [41]. BSA- and FITC-BSA-bound nanodiscs have slightly larger diameter than empty discs, which may be attributed to the BSA attachment onto the surface. Statistical comparison of the sizes of empty, BSA and FITC-BSA nanodiscs indicated no statistically significant differences. The hydrodynamic diameter of BSA is ranging from 6.6 to 7.9 nm [46], and thus not exhibited significant changes in size and leads to small increments in the nanodiscs sizes. However, the difference between the sizes of BSA-nanodiscs and FITC-BSA-nanodiscs with respect to the labeled FITC molecules is approximately 9.20%. According to published works, the size of nanocarrier systems has a significant role in drug delivery, as nanoparticles ($\leq$ 200 nm) can be accumulated in the tumor microenvironment due to the enhanced permeability and retention (EPR) effect and can offer long blood circulation because nanoparticles cannot easily recognized by the reticuloendothelial system (RES) [47].

Although there is data available for proteins charges, charge measurements of lipids are difficult to obtain due to their tendency to aggregate. Nanodiscs, however, present a promising strategy to overcome the lack of charge information. Nanodiscs act as a stable platform allowing electrophoretic charge measurements. Additionally, presence and negativity of the MSP belt that surrounds the lipid molecules can produce electrostatic repulsion [48–50]. The electrostatic repulsive force between the negatively charged molecules of the nanoparticles provides high stability to the colloidal solution and prevents nanoparticles from agglomerating [51]. In the current study, measuring the zeta potential allowed confirmation of albumin molecules binding to the nanodiscs, based on the changes in surface charge. All formed nanodisc exhibited negative potential due to the negatively charged molecules MSP, BSA and FITC, and the different discs are not significantly different from each other. Albumin is a negatively charged protein that showed adsorption activity on the neutral POPC model membranes and confirms the attractive interactions between the neutral lipid and the negatively protein. Furthermore, although POPC is a neutral lipid, the formed empty nanodiscs exhibited a negative charge due to the negative MSP proteins; POPC lipids contribute minimally to the measured charge. However, BSA-nanodiscs exhibited the lowest negativity while FITC-BSA-nanodiscs exhibited a slightly higher negativity than BSA-nanodiscs, which may be attributed to the negativity of both albumin and FITC molecules besides MSP. Significant zeta potential changes between different nanodiscs were not observed due to the use of neutral lipids. When POPC nanodiscs were bound to albumin, no significant changes in zeta potential values were observed. These results suggest that the interaction of albumin with zwitterionic lipid surfaces is driven by electrostatic interactions and the measured negative charge is mainly contributed by MSP molecules [50].

Particle physicochemical properties and drug release can influence bioavailability and pharmacokinetics properties. Particles size, shape and molecular weight significantly affect protein release [52]. Smaller particle size has a larger surface area, which enables the increase in surface area to volume ratio. Hence, reduction of surface area exposed to the dissolution medium results in a longer equilibrium time which contributes to sustained delivery of poorly soluble drugs [53,54]. Particle shape has also an important effect on release behavior, with spheres showing slower release than discs, rods, or slabs. Moreover, encapsulated proteins with small molecular weights exhibit short and quick release while large proteins show prolonged release [55]. Presence of albumin to control half-life of a drug may be considered as a key parameter in designing drug carriers to optimize pharmacokinetic and drug targeting properties. Hence, the generated nanodiscs may be exploited in the future to incorporate a membrane protein or hydrophobic molecules, thereby increasing the circulating half-life and bioavailability of small therapeutic agents.

The stability of the nanoparticles can be attributed to high zeta potential values and to protein release profile. As shown in Fig. 6, the stability of the formed nanodiscs was investigated indirectly by measuring release of MSP, BSA, and FITC-BSA from nanodiscs using UV-VIS spectrophotometry. Empty nanodiscs showed the highest stability among all developed nanosystems due to the highest zeta potential value and slowest protein release. Water molecules initiate release of protein molecules exposed at the surface of the matrix. This factor may play an important role in the rapid release of BSA that is more accessible while presence of FITC molecules may hinder fast water penetration or protein release. Moreover, besides the lower zeta potential, BSA-nanodiscs have relatively smaller diameters than FITC-BSA-nanodiscs based on hydrodynamic size. In nanomedicine, it is favorable to develop small particles because small size means deeper access to tissues and better surface area for reactions and interactions. The rate of interaction at or near the surface is also higher for the smaller particles. FITC may also increase the density of conjugated molecules, which could lead to a slower release rate. According to Determan [56], encapsulated FITC-BSA may also be released as insoluble aggregates that cannot be detected by the BCA assay.

Since nanodiscs can be used as a well-defined structure for single-molecule fluorescence applications, they offer an attractive system for *in vivo* [14] and *in vitro* [57,58] imaging, tracking the mechanism of reconstituted protein into lipid bilayer and formation of signaling complex in living cells. FITC fluorescent probe is widely used to monitor the delivery and bio-distribution of drug delivery systems. The cellular imaging technology can exploit nano-sized drug carriers to

evaluate the therapeutic efficacy by monitoring endocytosis and intracellular fate of drug-loaded particles [59]. Nanodiscs have great potential to be used in several applications involve imaging and as drug delivery system. Furthermore, the obtained results not only present BSA-bound nanodiscs as nanocarriers that can be exploited to incorporate hydrophobic agents but also as a vehicle that transports fatty acids to fatty acid-consuming cells such as cardiac muscle cells due to their fatty acid compositions. Moreover, FITC may influence the physicochemical properties and drug release. These observations in turn may impact the adsorption activity and evaluation of cellular uptake pathways which are important characteristics in biological studies that aim to understand cellular function or assess drug efficacy.

5. Conclusion

Nanodiscs are promising model of synthetic bioarchitectures that can be exploited in several research areas. Although nanodiscs have a broad range of applications in biophysical research, few studies have focused on their physical properties. In this study, we investigated the effects of a fluorescent probe on size, charge, and protein release characteristics of nanodiscs using albumin as a model protein. SDS-PAGE and DLS analyses show that the adsorption of BSA with or without FITC molecules leads to changes in the apparent hydrodynamic size and the electronegativity of the nanodiscs. The presence of the FITC isomer may affect the release of protein from the nanodiscs. This finding is critical for imaging studies as it may affect the adsorption activity and cellular uptake of nanodiscs, leading to different behaviors in physiological environments. The general protocol, analytical techniques and the findings described here may guide future studies on nanodisc function in the presence of a fluorescent or labeled molecule.

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