Engineering DNA-Grafted Quatsomes as Stable Nucleic Acid-Responsive Fluorescent Nanovesicles

Marianna Rossetti, Lorenzo Stella, Judit Morlà-Folch, Sara Bobone, Ariadna Boloix, Lorena Baranda, Danila Moscone, Mònica Roldán, Jaume Veciana, Miguel F. Segura, Mariana Köber,* Nora Ventosa,* and Alessandro Porchetta*

The development of artificial vesicles into responsive architectures capable of sensing the biological environment and simultaneously signaling the presence of a specific target molecule is a key challenge in a range of biomedical applications from drug delivery to diagnostic tools. Herein, the rational design of biomimetic DNA-grafted quatsome (QS) nanovesicles capable of translating the binding of a target molecule to amphiphilic DNA probes into an optical output is presented. QSs are synthetic lipid-based nanovesicles able to confine multiple organic dyes at the nanoscale, resulting in ultra-bright soft materials with attractiveness for sensing applications. Dye-loaded QS nanovesicles of different composition and surface charge are grafted with fluorescent amphiphilic nucleic acid-based probes to produce programmable FRET-active nanovesicles that operate as highly sensitive signal transducers. The photophysical properties of the DNA-grafted nanovesicles are characterized and the highly selective, ratiometric detection of clinically relevant microRNAs with sensitivity in the low nanomolar range are demonstrated. The potential applications of responsive QS nanovesicles for biosensing applications but also as functional nanodevices for targeted biomedical applications is envisaged.

1. Introduction

The responsive surface recognition properties of biological membranes have inspired a number of strategies for the design and manufacturing of biomimetic artificial vesicles. Synthetic vesicles are self-assembled colloidal particles composed of amphiphilic molecules enclosing a small aqueous compartment.[1–3] Such bilayer structures can be composed of natural or synthetic amphiphilic molecules ranging from lipids to surfactants and block copolymers.[4] Among these, lipid-based nanoparticles bear the advantage of being the least toxic for in vivo applications.[5] To sense and respond to their external environment, integration of specific binding sites within the vesicle structure or post-synthesis functionalization of the external layer with amphiphilic molecular probes have represented the main strategies so far.

Anchoring amphiphilic nucleic acid probes to lipid-based vesicles has found wide application in the production of new biomimetic hybrid structures like DNA-grafted liposomes,[6–8] artificial organelles,[9] and membrane pores.[10–12] Thanks to the selectivity and programmability of Watson-Crick base-pairing interactions, the embedding of synthetic DNA amphiphiles into artificial vesicles allowed the production of DNA-programmed functional materials,[3,14] with applications in bottom-up synthetic biology[15–17] and drug delivery.[18,19] In this regard, recently, supramolecular organization of multiple lipid-based vesicles mediated by nucleic acids self-assembled colloidal particles composed of amphiphilic molecules enclosing a small aqueous compartment.[1–3] Such bilayer structures can be composed of natural or synthetic amphiphilic molecules ranging from lipids to surfactants and block copolymers.[4] Among these, lipid-based nanoparticles bear the advantage of being the least toxic for in vivo applications.[5] To sense and respond to their external environment, integration of specific binding sites within the vesicle structure or post-synthesis functionalization of the external layer with amphiphilic molecular probes have represented the main strategies so far.

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adfm.202103511.

M. Rossetti, L. Stella, S. Bobone, L. Baranda, D. Moscone, A. Porchetta
Department of Chemical Science and Technologies
University of Rome Tor Vergata
Via della Ricerca Scientifica, Rome 00133, Italy
E-mail: alessandro.porchetta@uniroma2.it

J. Morlà-Folch, J. Veciana, M. Köber, N. Ventosa
Institut de Ciència de Materials de Barcelona
ICMAB-CSIC
Campus UAB, Bellaterra 08193, Spain
E-mail: mkober@icmab.es; ventosa@icmab.es

M. Roldán
Unitat de Microscòpia Confocal i Imatge Cellular
Servei de Medicina Genètica i Molecular
Institut Pediàtric de Malalties Rares (IPER)
Hospital Sant Joan de Déu
Esplugues de Llobregat, Barcelona 08950, Spain

M. Roldán
Institut de Recerca Sant Joan de Déu
Esplugues de Llobregat, Barcelona 08950, Spain

J. Veciana, M. Köber, N. Ventosa
Centro de Investigación Biomédica en Red CIBER-BBN
Madrid 28029, Spain

J. Veciana, N. Ventosa
Nanomol Technologies SL
Mòdul de Recerca B
Campus Universitari de Bellaterra
Cerdanyola del Vallès 08950, Spain
Acid interactions has been reported.\textsuperscript{[20,21]} Such structures can be held together by nucleic acid linkers in order to achieve fusion of multiple vesicle populations\textsuperscript{[22]} and construct compartmentalized chemical processes,\textsuperscript{[23]} and they can be used to study the physical properties of lipid-based self-assembled systems.\textsuperscript{[7,24]}

Several efforts have been devoted to engineer vesicles responsive to physical or molecular stimuli.\textsuperscript{[25–27]} In this regard, aggregation and fusion of lipid vesicles represent two dynamic processes that can be regulated by target binding to recognition elements anchored on the vesicle surface. These phenomena generally produce morphological changes of vesicles and transduce molecular recognition into detectable responses (e.g., changes in light scattering or sample turbidity). Unfortunately, the morphology of self-assembled soft phases can be strongly affected by small changes in the adhesion forces that are generally independent from the nature of the ligand.\textsuperscript{[28]} As a consequence, environmental changes can hamper the applicability of the vesicular systems for sensing applications.

Quatsomes (QSs) are an emerging class of highly stable small unilamellar vesicles of \textasciitilde 50–100 nm in diameter, formed by the self-assembly of ionic surfactants and sterols in aqueous media.\textsuperscript{[29,30]} Their high stability, that is, also in body fluids,\textsuperscript{[31]} unilamellarity and particle-to-particle homogeneity make them an attractive soft material for sensing applications. Furthermore, we recently demonstrated the possibility to confine hundreds of organic dyes in a single QS\textsuperscript{[32–34]} and even the simultaneous loading of a Förster resonance energy transfer (FRET) dye pair in QSs yielding ultra-bright nanovesicles, whose brightness compares with the most common quantum dots\textsuperscript{[35]} enabling their use for molecular detection and imaging. Finally, the cellular penetration capability of different types of fluorescent QSs in different cell lines has been also reported.\textsuperscript{[32–35]} In this respect, introducing responsiveness to external molecular inputs within fluorescent QS nanovesicles could pave the way for the rational design of multifunctional biomimetic vesicles capable of translating the recognition of target molecules into signal transduction for biosensing applications.

Motivated by the above considerations, here we anchor fluorescent cholesterol-modified DNA probes (i.e., F-miRNA probe) on dye-loaded QSs, to produce FRET-active nanovesicles responsive to clinically relevant nucleic acid targets such as microRNAs (miRNA), a class of small endogenous non-coding RNAs that can be used as diagnostic and prognostic markers in multiple human diseases.\textsuperscript{[36–38]} Considering that FRET typically occurs within a distance of 1–10 nm, the hybridization of specific sequences to DNA-grafted QSs may produce ratiometric FRET change to be used for miRNA detection. (Scheme 1). We demonstrate the versatility and robustness of this approach by functionalizing three types of dye-loaded QSs composed of different surfactants. We experimentally investigated photophysical properties of DNA-grafted FRET QSs and their responsiveness to miRNA targets.

2. Results and Discussion

2.1. Preparation of Fluorescent DNA-Grafted Nanovesicles

We designed three types of fluorescent QS nanovesicles, loaded with 1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (here named QS/DiI) prepared by a one-step method using the green technology referred to as depressurization of expanded liquid organic solution–suspension (DELOS-suspension) methodology.\textsuperscript{[39]} To tune the overall surface charge of the fluorescent nanovesicles, we employed either cationic or anionic surfactants, at an equimolar ratio with cholesterol (Chol). The 1:1 bimolecular pair formed by one surfactant and one cholesterol molecule in the following is termed “synthon”, since it represents the building block from which the vesicles are formed. In particular, tetradecyldimethylbenzylammonium chloride (MKC) has been employed as cationic surfactant to...
produce Chol/MKC nanovesicles with high positive surface charge (QS(+/+)/DiI, Figure 1A,B, Table S1, Supporting Information) than nucleic acid probes without the cholesteryl-TEG moiety is not able to bind to QS(−)/DiI vesicles (100 nM) in the presence of QS/DiI nanovesicles (Figure 1E, see Supporting Information for further details). QS(−)/DiI vesicles (red circles) show a lower affinity compared to QS(+/+)/DiI and QS(−)/DiI, in agreement with the contribution of electrostatic repulsions occurring between negatively charged surface and single-stranded nucleic acid.[46] As expected, the same DNA probe without the cholesteryl-TEG moiety is not able to bind to QS(−)/DiI nanovesicles (Figure S3, Supporting Information). To further demonstrate successful cholesterol-mediated DNA anchoring to QS/DiI nanovesicles, we also performed polyacrylamide gel electrophoresis assays on QSSs functionalized with different concentrations of AT425-miR-21 probe. As expected, cholesteryl-TEG-modified oligonucleotides interact more efficiently with lipid-based membrane surfaces (Figure 1F and Figure S4, Supporting Information) than nucleic acid probes without the cholesteryl moiety, for all the systems tested.

2.2. Photophysical Properties of FRET Active DNA-Grafted QS

The FRET pair of interest is composed of the amphiphilic cyanine dye DiI (1,1'-Diocadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate, embedded in the QS membrane) and the far-red
fluorophore Alexafluor 647 (conjugated with the nucleic acid probe at the 5'-end), acting as donor and acceptor, respectively. It should be noted that we have also investigated an additional FRET pair, with the Dil fluorophore functioning as acceptor in the Q5 membrane, and ATTO425 (conjugated to the nucleic acid) as FRET donor. However, the FRET efficiency observed with the ATTO425-Dil pair was significantly lower than using Dil-AlexaFluor647 (Figure S5, Supporting Information). Moreover, the ATTO425-Dil configuration did not provide significant FRET efficiency for all Q5 formulations (see Figure S5 and S6, Supporting Information, for further information). Thus, in the following, we will focus on Q5/Dil nanovesicles functionalized with an Alexafluor 647 (AF647)-labeled miR-122 probe (i.e., AF647-miR-122 probe), as a first model system to demonstrate FRET active Q5s responsive to a miRNA target.

Steady-state fluorescence emission of Q5/Dil nanovesicles in the absence of AF647-miR-122 probe show identical emission profiles for Q5(+/+)/Dil and Q5(+/+)Dil, with the characteristic features of Dil emission, with a maximum of emission at ≈565 nm and a shoulder at ≈605 nm (Figure 2A, green and blue lines, and Table S2, Supporting Information). The enhanced intensity of the emission band at ≈595 nm combined with the additional shoulder at ≈645 nm in Q5(−)/Dil can instead be indicative of aggregation-induced emission phenomena of the cyanine dyes in the lipid nanovesicles (Figure 2A, red line). Absorption and excitation spectra seem to confirm the hypothesis of fluorescent aggregates in Q5(−)/Dil nanovesicles (Figures S7 and S8, Supporting Information).[45]

Fluorescence quantum yields (Φf) of Dil embedded in the three Q5s (Tables S2 and S3, Supporting Information) confirm recently reported values.35] Upon functionalization with AF647-miR-122 probe, fluorescence emission spectra indicate the occurrence of energy transfer in the three Q5/Dil systems (Figure 2C). Upon excitation at 520 nm, two bands appear, with maxima at ≈565 and ≈675 nm corresponding to the Dil and AF647 fluorescence emission, respectively. The fluorescence intensity of the donor (Dil) decreases in the presence of the acceptor (AF647), resulting in FRET efficiencies (ΕFRET) ranging from 80% (Q5(+/+)Dil/AF647-miR-122 probe) to 25% (Q5(−)/Dil/AF647-miR-122 probe, see Table S3, Supporting Information). Excitation spectra obtained when probing acceptor emission at λem = 750 nm in the presence and in the absence of Q5/Dil nanovesicles clearly confirm that changes in fluorescence emission can be ascribed to FRET mechanism in the three systems (Figure 2B and Figure S9, Supporting Information). In addition, a significant red shift of ≈10 nm in the maximum of AF647 emission for Q5(+/+)Dil/AF647-miR-122 probe (green line) and Q5(+/+)Dil/AF647-miR-122 probe (blue line) compared to free miR-122 probe (dashed black line) in solution is reported, indicating that positively charged nanovesicles more strongly interact with nucleic acid probes. Of note, FRET efficiency increases as a function of acceptor concentration (AF647), reaching its maximum value in the presence of 200 nM of AF647-miR-122 (Figure S10, Supporting Information). All three nanovesicle systems protect the oligonucleotide probe from nuclease digestion, although to a different extent (Figure S11, Supporting Information), showing no significant degradation when the amphiphilic probe is anchored to highly positively charged Q5 nanovesicles, and only similar partial degradation when anchored to

Figure 2. Emission spectra of plain and AF647-miR-122 probe functionalized Q5/Dil nanovesicles. A) Normalized emission spectra of Q5(+/+)Dil (green), Q5(+/+)Dil (blue), Q5(−)/Dil (red) by exciting Dil embedded in the particles (λex = 488 nm). B) Normalized emission (left) and excitation fluorescence spectra (right) of Q5(+/+)Dil/AF647-miR-122 probe (green), Q5(+/+)Dil/AF647-miR-122 probe (blue), Q5(−)/Dil/AF647-miR-122 probe (red) and AF647-miR-122 probe free in solution (dashed black line). Emission spectra are obtained by exciting AF647 at a fixed wavelength (λex = 520 nm). Excitation spectra are recorded by fixing the emission at λem = 750 nm and moving excitation from 550 to 700 nm. C) Emission spectra of Q5(+/+)Dil (left), Q5(+/+)Dil (middle), Q5(−)/Dil (right) nanovesicles in the absence (grey) and in the presence of AF647-miR-122 probe (200 nM, black line), showing quenching of donor fluorescence (Dil) and increase of acceptor (AF647) emission.
QS(+) and QS(−). This can be ascribed to the high flexibility of single-stranded DNA which can help maximize the electrostatic attraction of negatively charged phosphate backbone to the positively charged vesicle surface, resulting in more flattened configurations of DNA that make the probe inaccessible to nuclease digestion. The similar behavior of QS(+) and QS(−), instead, could be ascribable to the surface charge of QS(+), just slightly positive (≈15 mV) that establishes only weak electrostatic interactions with the negatively charged phosphate backbone, thus not able to completely hinder the nuclease activity of the enzyme towards the DNA probe.

### 2.3. miRNA Detection using DNA-Grafted QSs

To explore the use of the FRET platform for sensing applications, we tested the possibility of detection of mature miRNAs. We expect the single strand to duplex transition of the miRNA probe to induce a significant probe elongation, resulting in an increase of the average distance between FRET donor and acceptor, and a consequent decrease in FRET efficiency (Figure 3A). The hypothesis of a measurable FRET change in the presence of target miRNA relies on the theoretical calculation of the Förster radius (R0) in the three QS/DiI/AF647-miR-122 probe systems (R0 is equal to 5.6 ± 0.1 nm, 5.3 ± 0.1 nm, and 5.6 ± 0.1 nm for QS(+), QS(+) and QS(−), respectively; the estimated surface density of acceptor molecules and the expected change in the conformation upon hybridization of target miRNA (the persistence length of a 22 base long DNA-RNA hybrid is ≈7 nm) (see Supporting Information and Figure S12, Supporting Information).

Ratiometric FRET changes for the three QS/DiI/AF647-miR-122 probe systems are obtained in the presence of complementary target miR-122 (black curves, Figure 3B). Indeed, by adding increasing concentrations of miR-122 we observe an increase in the DiI emission and a consequent decrease of the emission associated with AF647, resulting in a decrease of the FRET efficiencies calculated from the donor’s (DiI) steady-state fluorescence (see Table S2, Supporting Information). The three ratiometric detections are quantitative in the nanomolar range of target concentration and highly specific, showing no significant FRET changes in the presence of non-specific target (Figure 3C and Table S4, Supporting Information). More specifically, the target concentrations at which the observed signal change is half the maximum signal change (K1/2) are 540 ± 30 nM, 220 ± 10 nM, and 91 ± 9 nM for QS(+), QS(+) and QS(−), respectively. As expected, no specific miRNA target detection is achieved in absence of the hydrophobic cholesterol moiety on the miR-122 probe (Figure S13, Supporting Information), as these probes merely interact electrostatically with the nanovesicle surface and can therefore be displaced from the QS surface at increased concentrations of nucleic acids of arbitrary sequence. To improve the overall sensitivities, we also tested lower concentrations of miR-122 probe anchored on the QS/DiI surface that coherently results in lower limit of detection at the cost of a decrease in the change of ratiometric

![Figure 3](image-url)

**Figure 3.** miRNA detection by using FRET-active DNA-grafted QS nanovesicles. A) Schematic representation of the platform for miRNA sensing by using QS/DiI/AF647-miR-122 probe. In the absence of miR-122, AF647-miR-122 probe is anchored on the QS surface in a random coil conformation that allows AF647 (acceptor of the FRET couple) to be in close contact with DiI (donor of the FRET couple) loaded in QS nanovesicle, resulting in a high FRET efficiency. In the presence of the miR-122 target, the binding induces a conformational change of the AF647-miR-122 probe resulting in an increase of the average donor-acceptor distance and lower FRET efficiency. B) Emission spectra of QS(+)DiI (left), QS(+)DiI (middle), QS(−)DiI (right) functionalized with 200 nM of AF647-anti-miR-122 probe in the absence (green, blue, and red lines, respectively) and in the presence of miR-122 (i.e., 300 nM, black lines). C) Binding curves obtained by adding increasing concentration of the specific (miR-122, black) or non-specific target (miR-21, grey; miR-31, light blue; miR-143, fuchsia; miR-145, orange) to a solution containing QS/DiI/AF647-miR-122 probe. From left to right the binding curves related to QS(+) and QS(−). All the experiments are performed in PBS buffer (94 mM NaCl, 3.1 mM Na₂HPO₄, 0.9 mM NaH₂PO₄, pH 7.4), at 25 °C by exciting vesicle-embedded DiI (λex = 520 nm).
FRET (Figure S14, Supporting Information). As expected, by decreasing the concentrations of anti-miR-122 from 200 to 30 nM, occupancy is no longer defined by the true affinity of the nucleic acid probe or the concentration of free target in solution but instead by the total number of target molecules in the sample relative to the total number of probes on the sensor surface (i.e., ligand-depletion conditions).

3. Conclusions

In this work, we developed and characterized a new platform based on highly stable and bright FRET-active nanovesicles that are responsive to clinically relevant nucleic acid targets. For this purpose, fluorescent amphiphilic nucleic acid probes were anchored on dye-loaded QS nanovesicles through a cholesterol moiety. We demonstrate the specific and ratiometric detection of miRNA with different QS systems, exhibiting either positive or negative surface charges or a functionalization with polyethylene glycol. The possibility to load QS nanovesicles with different donor/acceptor fluorescent pairs and different probes might also allow to achieve multiplex simultaneous detection of molecular targets in an orthogonal way. Indeed, the proposed strategy could be easily adapted to the detection of different biomarkers, by simply using rationally designed amphiphilic structure-switching DNA probes. Although, the overall sensitivity of the platform is still not high enough for clinical monitoring of miRNA levels in vitro, further engineering of both the QS nanovesicle and DNA probe will allow to improve the signal transduction and the overall sensitivity. We also expect to achieve a bioimaging platform for the detection of a wide range of nucleic acids and other clinically relevant molecules (using aptamers) in complex body fluids or directly in cells, thanks to the capability of DNA-grafted QSs for intracellular delivery (Figures S15 and S16, Supporting Information). Besides the sensing applications, the functionalization of lipid vesicles with amphiphilic nucleic acids can provide a means to rationally introduce responsiveness to environmental cues into artificial lipid-based protocells, which represents one of the main challenges in synthetic biology and biomimetic biotechnology. This approach can thus open horizons in the field of multi-functional hybrid nanovesicles for cell imaging and theranostics, since the incorporation of functional amphiphilic probes can be used to improve the targeting ability and help increase the efficacy of therapeutic applications.

4. Experimental Section

Reagents and Materials: Sodium chloride (NaCl), sodium dihydrogen phosphate (NaH₂PO₄), disodium hydrogen phosphate (Na₂HPO₄), magnesium chloride (MgCl₂), 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris base), boric acid, ethylenedinitrilo)tetraacetic acid (EDTA), acrylamide/bis-acrylamide 30% solution, ammonium persulfate (APS), N,N,N′,N′-tetramethyl ethylenediamine (TEMED), tetraethylmethylenbenzylammonium chloride (MCK), cholesteryl-polyethylene glycol 1000 (Chol-PEG1000) were purchased from Sigma-Aldrich, Italy and used without any further purifications. Orange DNA Loading Dye (6x) and O’Range Ruler 5 base pair DNA Ladder were purchased from Thermo Fisher Scientific Italia. 5-Cholestren-3β-ol (Chol, purity 95%) was purchased from Panreac (Barcelona, Spain).

© 2021 The Authors. Advanced Functional Materials published by Wiley-VCH GmbH
performed (reported Figures 2E and Figure S3, Supporting Information), by adding increasing concentrations of synthon to a solution containing fixed amount of AT425-miR-21 probe (100 nM). Nuclease experiments were performed by adding 10 µg of DNAse I in 45 µL of solution containing 30 nM of miR-21 probe dual labeled with and without QS nanovesicles (synthon equal to 26 µM). Kinetic curves were performed with excitation at 445 nm and acquisition at 480 nm, bandwidths of 5 nm in excitation and emission.

Absorption Experiments: All the absorbance measurements were performed in 100 µL solution of Na2HPO4 (3.1 mM) + NaH2PO4 (0.9 mM) + NaCl (94 mM) at pH 7.4 at 25 °C, using cuvette Hellma quartz cells (1 cm pathlength) and a Varian Cary 50 UV–Vis Spectrophotometer. To determine the concentration of DiI entrapped in the Q S particles, the UV–Vis absorbance of the dye was measured by diluting the samples in ethanol, in order to dissociate QS membranes, until an absorbance value of 0.1–0.3 was obtained. The concentration of the dye was determined using Lambert–Beer law \((ε_{exc}^{450 \text{nm}} \text{ in EtOH} = 140,000 \text{ M}^{-1}\text{cm}^{-1})\).\(^{[47]}\)

Native Polyacrylamide Gel Electrophoresis (PAGE) Assays: Native PAGE experiments were performed by using 12% polyacrylamide (29:1 acrylamide/bisacrylamide) in TBE 1× buffer (1 M Tris, 0.9 M boric acid, and 0.01 M EDTA) at pH 8.3 for 3 h. After 30 min of staining in SYBR gold (Invitrogen) dissolved in a TBE 1× buffer at pH 8.3, the gel was scanned on a cell electrophoresis unit (Bio-Rad) at room temperature, at a constant voltage of 100 V, using TBE 1× buffer (0.1 M Tris, 0.09 M boric acid, and 0.001 M EDTA) at pH 8.3 for 3 h. After 30 min of staining in SYBR gold (Invitrogen) dissolved in a TBE 1× buffer at pH 8.3, the gel was scanned by a Gel Doc XR+ system (Bio-Rad).

Cryogenic Transmission Electronic Microscopy: Cryogenic transmission electronic microscopy (cryoTEM) images was acquired with a JEOL JEM microscope (JEOL JEM 2100, Tokyo, Japan) operating at 200 kV under low-dose conditions. The sample was deposited onto the holey carbon grid and then it was immediately vitrified by rapid immersion in liquid ethane. The vitrified sample was mounted on a cryo-transfer system (Gatan 626) and it was introduced into the microscope. Images were recorded on a CCD camera (Gatan Ultrascan US1000) and they were analyzed with the Digital Micrograph 1.8 software.

Dynamic Light Scattering (DLS): The authors measured the size, polydispersity index and ζ-Potential of all the nanovesicles produced using a Zetasizer Nano ZS (Malvern Instruments) with an incident light (488 nm) in the presence and in the absence of the acceptor AF647, respectively.

Loading of miRNA Probe on QS Surface (%): For each concentration of QS nanovesicles, loading was estimated as follows

\[
\text{Loading(%)} = \frac{F_{(Q5/Di)} - F_{(AT425-miR-21 probe)}}{F_{(max)} - F_{(AT425-miR-21 probe)}} \times 100
\]

where \(F_{(Q5/Di)}\) is the fluorescence signal of AT425-miR-21 probe at the maximum \(\lambda_{nm} \text{ max} = 485 \text{ nm}\) in the presence of Q S/ Di nanovesicles and \(F_{(AT425-miR-21 probe)}\) is the fluorescence signal of AT425-miR-21 probe in the absence of Q S nanovesicles.

\[\text{Fluorescence (Donor)} = \frac{\text{Fluorescence (Acceptor)}}{\text{Rat.FRET =}} \]

The binding curves reported in Figures 3C and Figure S14, Supporting Information, were fitted with the following four parameter logistic equation:

\[
\text{Rat.FRET(T)} = \text{Rat.FRET}_0 + \left(\frac{T_0 - \text{Rat.FRET}_0}{T^{\text{Hill}} + K_{\text{Hill}}^{\text{02}}}ight)
\]

where Rat. FRET (T) is the FRET value in the presence of different concentration of target; Rat. FRET0 = ratiometric FRET value in the absence of target; \(T\) = target concentration; Rat. FRET = ratiometric FRET in the presence of saturating concentration of target; \(K_{\text{Hill}} = \text{the observed equilibrium concentration at half-maximum signal gain}; n_{\text{Hill}} = \text{the Hill coefficient}.

\[\text{FRET Efficiency and Fö rster Radius: FRET efficiencies were calculated from the decrease in donor’s (DiI) steady state fluorescence, by using the following equation}
\]

\[
E = 1 - \frac{F_2}{F_0}
\]

where \(F_2\) and \(F_0\) are the fluorescence emission values of DiI at 567 nm, in the presence and in the absence of the acceptor AF647, respectively. Förster radii were calculated for the three different QS samples using the following equation:

\[
R_0^2 = 8.75 \times 10^{-2} \frac{2}{n^2} \phi_0 J
\]

where \(\phi_0\) is the quantum yield of the donor, in the absence of the acceptor, \(n\) is the refractive index of the medium (water, 1.333), and \(J\) is the spectral overlap integral calculated as

\[
J = \frac{1}{\lambda_D} e_{\lambda_A} (\lambda) \lambda^4 d\lambda
\]

where \(\lambda_D\) is the donor (Q5/Di) emission spectrum normalized to an area of 1, and \(e_{\lambda_A}\) is the acceptor (AF647) molar extinction coefficient (expressed as M\(^{-1}\) cm\(^{-1}\)) and the wavelength is expressed in nm.

Cell Lines: HEK293T were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and stored in liquid nitrogen. Upon resuscitation, HEK293T cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Thermo Fisher Scientific, Waltham, Massachusetts, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS) South America Premium (Thermo Fisher Scientific), 100 U mL\(^{-1}\) penicillin, 100 µg mL\(^{-1}\) streptomycin (Thermo Fisher Scientific) and 5 µg mL\(^{-1}\) plasmocin (InvivoGen, San Diego, CA, USA). All cultures were maintained at 37 °C in a saturated atmosphere of 95% air and 5% CO2. HEK293T cells were tested for mycoplasma contamination periodically.
Confocal Imaging: HEK293T cells were seeded at 5.5 × 10^4 in 8-wells Ibidi chamber slides coated with poly-L-lysine (#80 824; Ibidi) in DMEM supplemented with 10% FBS without antibiotics. DNA-grafted QS were prepared by the combination of 10 μg mL^{-1} QS(+)+ and 50 nM of dual labeled miR-21 probe or 10 μg mL^{-1} QS (+)/DiI and 50 nM of AF647-miR-122 probe labeled with Alexa647 and incubated with HEK293T cells for 24 h. The next day, cellular media was changed and images of five random fields were acquired using a Leica TCS SP8 microscope equipped with a white light laser and Hybrid spectral detectors (Leica Microsystems GmbH, Mannheim, Germany). The confocal images were performed using a HC x PL APO 63 × 1.4 oil immersion objective and five random fields were acquired using a Leica TCS SP8 microscope equipped with a white light laser and Hybrid spectral detectors (Leica Microsystems GmbH, Mannheim, Germany). The confocal images were performed using a HC x PL APO 63 × 1.4 oil immersion objective and five random fields were acquired using a Leica TCS SP8 microscope equipped with a white light laser and Hybrid spectral detectors (Leica Microsystems GmbH, Mannheim, Germany).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements
This work was financially supported by the European Union’s Horizon 2020 research and innovation program within the Marie Skłodowska-Curie grant agreement “Nano-Oligo Med” (No 778133), Ministry of Science and Innovation (MINECO), Spain, through the “MOL4BIO” project (PID2019-105622RB-I00) and by Instituto de Salud Carlos III (DT2020-000018), Italian Ministry of University and Research (Project of National Interest, PRIN, 2017Y2PAB8_004) through the project “Cutting Edge Analytical Chemistry Methodologies and Bio-Tools to Boost Precision Medicine in Hormone-Related Diseases”. M.R. was supported from a MINECO through the Severo Ochoa Programme for Centers of Excellence in R&D (SEV-2015-0496 and CEX2019-000917-S). Quatsome production and their physicochemical characterization has been performed by the Biomaterial Processing and Nanostructuring Unit (U6) of the ICTS “NANBIOSIS”, a unit of the CIBER-BBN located at the Institute of Materials Science of Barcelona (ICMAB-CSIC).

Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Research data are not shared.

Keywords
biosensing, fluorescence, nanovesicles, responsive nanomaterials

Received: April 13, 2021
Revised: July 16, 2021
Published online: August 11, 2021
