Synthesis, characterization, and biological applications of semiconducting polythiophene-based nanoparticles

Mattia Zangoli | Francesca Di Maria

Consiglio Nazionale Ricerche CNR-ISOF and Mediteknology srl, Bologna, Italy

Correspondence
Francesca Di Maria, Consiglio Nazionale Ricerche CNR-ISOF and Mediteknology srl, via P. Gobetti 101, I-40139 Bologna, Italy.
Email: francesca.dimaria@isof.cnr.it

Abstract
In recent years, polythiophene-based nanoparticles (PT-NPs) have attracted increasing attention because of their outstanding characteristics deriving from a wealth of properties such as charge conduction in the oxidized/reduced states, light absorption/emission at an appropriate wavelength, geometrical adaptability, thermal and chemical stability, and solubility in common solvents. Furthermore, the great synthetic flexibility of the thiophene core has allowed the engineering of a multitude of nanomaterials with made-to-order properties. This review is focused on the synthesis and characterization of aqueous PT-NPs and their biological applications. In particular, both in vitro and in vivo bioapplications will be presented, in which thiophene-based nanomaterials act as photoactuators, that is, as exogenous components capable of transforming a primary stimulus of light into a highly spatiotemporally resolved secondary stimulus able to alter cell’s physiological functions. Furthermore, examples of applications of PT-NPs in biomedicine, such as photodynamic therapy and photothermal therapy, will be discussed.

KEYWORDS
in vitro and in vivo bioapplications, light-activated bioactuators, nanoparticles preparation & characterization, photodynamic therapy, photothermal therapy, water-dispersed polythiophene-based nanoparticles

1 | INTRODUCTION

Over the past two decades, research on thiophene-based materials (TpM) has seen a very rapid expansion. Its multidisciplinary character foresees their implementation as active materials both in organic electronics—such as organic solar cells, organic field-effect transistors, and organic light-emitting diodes (OLED)—and biological applications.1–9 In the vast Pantheon of conjugated organic polymers, TpM play a leading role owing to their peculiar functional properties, chemical robustness, biocompatibility, and versatility.10–13 Most of their characteristics are the result of their delocalized electronic structure, which among other things determines a strong light absorption because of intense \( \pi-\pi^* \) transitions. Furthermore, the facile functionalization of the...

Abbreviations: DNA, deoxyribonucleic acid; HOMO, highest occupied molecular orbital; LUMO, lowest unoccupied molecular orbital; RNA, ribonucleic acid; THF, tetrahydrofuran

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. VIEW published by John Wiley & Sons Australia, Ltd and Shanghai Fuji Technology Consulting Co., Ltd, authorized by Professional Community of Experimental Medicine, National Association of Health Industry and Enterprise Management (PCEM)

wileyonlinelibrary.com/journal/viw2 
https://doi.org/10.1002/VIW.20200086
Figure 1. Functionalization of thiophene ring (left) and general structures of TpM homopolymers and copolymers (right).

The purpose of this review is to provide an overview of the synthesis, chemical-physical properties, and in vivo validations of PT-NPs to highlight and raise the interest in these extraordinary nanomaterials. The different methodologies for the synthesis and characterization of PT-NPs will be briefly presented. Then, in subsequent chapters, the most recent biological applications that exploit PT-NPs as light-activated bioactuators will be described including the most promising and advanced applications in phototherapy.

2 | SYNTHESIS OF SEMICONDUCTING PT-NPs

PT-NPs can be prepared through either direct or postpolymerization techniques. In the first case, NPs are formed in situ during polymer synthesis through a bottom-up approach, in the latter they are prepared after polymer preparation by a top-down approach. However, postpolymerization techniques are currently considered as the most versatile methods to prepare NPs suspended in water, a crucial requirement in biological applications. Moreover, a wider variety of structurally modified and functionalized polythiophenes with higher levels of purity can be used. Postpolymerization techniques include nanoprecipitation/reprecipitation and miniemulsion methods (Figure 2).

2.1 | Nanoprecipitation method

Generally, in the nanoprecipitation method, the conjugated polythiophene (PT) derivative (≈10⁻⁴ M) is first dissolved in a good solvent and then dispersed into a poor solvent, that is, water, under vigorous stirring or ultrasonication (Figure 2). The solvent employed to dissolve the polymer has to be miscible with water (e.g., THF, CH₃CN), therefore, once the polymer solution encounters water, NPs are instantaneously formed as a result of a combination of hydrophobic and π–π interactions, which force the exclusion of water to minimize the contact area of the polymer. The residual organic solvent can be removed by evaporation under reduced pressure or by dialyzing the colloidal suspension. Chart 1 reports the chemical...
**CHART 1**  Selected examples of PTs able to self-assemble into NPs via nanoprecipitation

**FIGURE 2**  Illustration of PT-NPs preparation via nanoprecipitation and miniemulsion methods
structures of different PTs that have been reported to form NPs following this methodology.\textsuperscript{37–49}

The nanoprecipitation method, however, commonly results in NPs batches with a large degree of polydispersity (PDI), that is, a wide NPs size distribution. Nevertheless, through differential centrifugation it is possible to select, within a sample, different fractions having more homogeneous dimensions and a reduced PDI index. For example, poly-3-hexylthiophene (P3HT) nanoprecipitated from a THF solution (10\textsuperscript{−4}) forms water dispersed NPs ranging from 100 to 600 nm, with excellent colloidal stability in the absence of surfactants, and with PDI values higher than 0.2. Fractions of NPs of different size can be conveniently separated through fractional centrifugation resulting in a 10-fold lowering of the PDI index (\textasciitilde 150 nm and \textasciitilde 350 nm sized NPs with PDI of 0.015 and 0.03, respectively).\textsuperscript{30} On the other hand, the size and the internal supramolecular organization of the polymer in NPs, which in turn influence photophysical and biological properties, can be controlled by intervening on one of the different parameters implicated in the nanoprecipitation process, such as concentration, PDI and regioregularity of the polymer, solvent volume, dripping time, stirring speed, ultrasonication intensity, and temperature.\textsuperscript{35,51} For instance, it has been experimentally proved that the size of PT-NPs can be reduced: (a) lowering the concentration of the starting polymer solution, (b) improving the characteristic of the polymer in terms of PDI and regioregularity, (c) favoring the mixing, and (d) using a greater solvent volume.\textsuperscript{25,35,52} Alternatively, the functionalization of the starting polymer through the introduction of solvophilic/phobic moieties in a specific ratio allows adding a further level of control over NPs size.\textsuperscript{38,53} Recently, our group demonstrated that by tuning the hydrophilicity/hydrophobicity balance in P3HT, through the selective introduction of oxygen atoms in the thiophene units of the polymer backbone, it is possible to form stable NPs, whose size can be downscaled to 5 nm forming polymer dots, that is, NPs consisting predominantly of a single polymer chain (Figure 3).\textsuperscript{38}

Typically, colloidal solutions obtained through nanoprecipitation are quite stable, even if amphiphilic surfactants or encapsulating matrices, such as sodium dodecyl sulfate (SDS), Triton X-100, and PEG-functionalized lipid, can be used to increase water dispersibility and to aid in stabilizing the suspension.\textsuperscript{54–56} During NPs formation, the hydrophobic components of the surfactant/matrix are embedded into the polymeric NPs core, while the hydrophilic segments remain oriented toward the aqueous environment, that is, around the NPs shell, as a consequence of their strong interactions with water. Changing the nature of the stabilizer and its concentration can impact on the size and surface chemical properties of the PT-NPs.\textsuperscript{32,57} Moreover, stabilizers can also influence the solubility, toxicity, and reactivity of the NPs and further support surface functionalization.\textsuperscript{58} For example, Guo et al report that polythiophenes can be nanoprecipitated together with amphiphilic matrices, that is, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-((amino [polyethylene glycol]-2000) (DSPE-PEG2000) and DSPE-PEG200-Maleimide, under ultrasonication. The role of the matrices is mainly to increase the solubility of the NPs. Furthermore, because of the presence of reactive groups, that is, maleimide, it is possible to covalently bind on their surface, via Michael addition reaction, a specific cyclo(Arg-Gly-Asp-D-Phe-Lys(mpa)) (c-RGD) peptide able to target specific receptors overexpressed in endothelial cells of the brain tumor, thus significantly enhancing the NPs uptake within the tumor.\textsuperscript{47}
The presence of chemical functional groups on the surface of NPs is an important characteristic for in vitro and in vivo bioapplications, as it affects the interaction with the environment, cytotoxicity, biodistribution, and allows to mark a specific target with high selectivity.\(^{59-61}\) The presence of charges on NPs surface has been proved to markedly influence cell internalization.\(^{59-61}\) Yin et al prepared PT-NPs decorated on the surface with positive charges achieving efficient cellular uptake and at the same time excellent biocompatibility, control over size, and high colloidal stability because of electrostatic repulsion. In their study, a polythiophene derivative was first nanoprecipitated with poly(styrene maleic anhydride) (PSMA) forming carboxyl-decorated NPs with a large negative surface charge. Then, a cationic polymer—that is, poly(L-lysine), a polymer widely employed for stem cell imaging—was allowed to spontaneously self-assemble on the negatively charged surface of the NPs, thus converting it into a positively charged one.\(^{44}\) The functionalization of PT-NPs with specific moieties can be also conducted before NPs formation and without employing matrices by incorporating chemically reactive groups and/or specific ligands such as amino, carboxylic, active esters, peptides, and proteins, by direct conjugation to the starting material through covalent bonding. For instance, we recently reported that the introduction of N-succinimidyl-ester groups at the terminal position of the side alkyl chains of P3HT allows obtaining NPs with a reactive shell capable of covalently binding to primary amine groups present in amino acids, peptides, and proteins through the formation of amidic bonds, thus allowing NPs to dock to the cellular membrane of human embryonic kidney (HEK-293) cells.\(^{41}\)

As presented in the aforementioned examples, PT-NPs can be easily delivered to biological systems as a water suspension, and the functionalization of their surface enables to control their localization and biodistribution. Moreover, by operating in aseptic condition, that is, by leading the entire preparation under a laminar flow hood using sterilized laboratory glassware and water, it is possible to obtain sterile NPs, a crucial prerequisite for their in vivo application.\(^{50}\)

### 2.2 Miniemulsion method

In the miniemulsion method, the starting polymer is dissolved in a water-immiscible solvent (e.g., CH\(_2\)Cl\(_2\), CHCl\(_3\), toluene), and then dispersed into water in the presence of a surfactant under vigorous stirring or ultrasonication (Figure 2). The vigorous mixing of the two immiscible phases induces the formation of small droplets of the polymer solution surrounded by water that have to be stabilized by a surfactant to avoid their rapid coalescence.\(^{62}\) The organic solvent is then evaporated, and the droplets collapse, thus forming stable water suspended NPs, and the excess of surfactant is finally eliminated by dialysis. In analogy to nanoprecipitation method, the resulting NPs size depends on the nature and concentration of the polymer and surfactant employed, stirring methodology, emulsion temperature, selected organic solvent, etc. Miniemulsion techniques are commonly utilized when PTs are not fully soluble in a water-mixable solvent.\(^{63-65}\) In general, the obtained NPs have a size in the range 30-500 nm, and a layer of surfactants always stabilizes their surface. For instance, P3HT-NPs can be prepared by dissolving a few milligrams (2 mg/mL) of highly regioregular polymer in chloroform, toluene, or cyclohexane, that is, the organic phase of the emulsion, and by combining this solution with water containing a surfactant, generally SDS or poly(ethylene glycol) methyl methacrylate (PEGMA), in a concentration higher than its critical micellar concentration. Changing the nature of organic solvent deeply affects the size of P3HT-NPs, indeed, NPs made starting from a chloroform solution are consistently larger (142 nm, PDI 0.26) than those obtained from a toluene solution (124 nm, PDI 0.27).

The surfactant employed and the conditions selected to prepare the dispersion can also modify intramolecular aggregation.\(^{66}\) For example, it was found that P3HT-NPs prepared using a mixture of organic solvents, that is, chloroform/toluene, display a higher structural order compared to those prepared using chloroform alone, determining a variation of the optoelectronic properties of the NPs.\(^{67,68}\) Size and crystal phase may affect colloidal stability and consequently biological availability of PT-NPs as a result of different surface properties of the nanomaterials including their interactions with biomolecules.

### 3 CHARACTERIZATION OF SEMICONDUCTING PT-NPs

Various techniques have been employed to characterize the size, crystallinity, elemental composition, and the many other physical properties of PT-NPs (Figure 4).\(^{69}\)

Generally, a combined characterization approach is employed to evaluate their properties. The morphological features and the dimensionality of the nanostructure are of great interest since they strongly influence most biological aspects such as cellular uptake, cytotoxicity, and biodistribution.\(^{70-72}\) The size, size distributions, and morphology characteristics are usually determined by combining dynamic light scattering (DLS) and microscopic techniques such as atomic force microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). By DLS measurements, it is possible to obtain
information regarding the average size, that is, the hydrodynamic radius and PDI index of the sample. DLS is one of the most employed techniques as it presents advantages such as short measurement time, high sensitivity to aggregates, and noninvasiveness and provides a good statistical representation of the NPs sample.73–76 Additionally, DLS allows determining the zeta potential (ζ) of NPs suspension, an important parameter which is indicative of the surface charge of the nanoparticle and gives information on the stability of the suspension.76,77 Colloids with a high ζ (absolute values greater than 30) are electrostatically stabilized, and their dispersions resist aggregation and thus have a long shelf life. For instance, P3HT-NPs even in the absence of surfactant show high ζ values, in the range between −30 mV and −40 mV, indicative of a high stability degree.50 The functionalization of NPs surface can influence the ζ value and its sign. For example, the use of surfactants or the postfunctionalization of P3HT-NPs surface with oxygen can considerably increase the ζ values (>−60 mV).38,67 Also, the introduction of cationic groups into the PT backbone allows to obtain NPs with a positive charge on the shell leading to a sign reversal in the ζ value (>+50 mV).42 Similar to DLS, microscopic techniques consent to measure NPs size as well as their size distribution—although samples are analyzed after solvent evaporation, often causing discrepancies in sizing among the techniques—but they also allow to investigate their morphology and perform a fast check of the elemental composition of the NPs, if combined with energy-dispersive X-ray spectroscopy (EDX).76

The evaluation of the surface properties and structural characteristics of NPs is also of primary importance to study the composition, aggregation, and bonding nature of the constituting nanomaterial.78 The surface chemistry of NPs plays a pivotal role in dictating their behavior both in vitro and in vivo.79 SEM/TEM-EDX and Zeta potential measurements, as aforementioned, can give information on the elemental composition and charge of the NPs surface, respectively; however, other techniques such as nuclear magnetic resonance (NMR) and X-ray photoelectron spectroscopies (XPSs) are fundamental to fully elucidate the NPs’ surface composition and properties. For example, NMR spectroscopy has been employed to study the stability/reactivity in deuterated water of NPs.
functionalized on their surface with amine-reactive-N-succinimidyl ester (NHS) groups. The NHS group can bind covalently to the primary amine moieties present in amino acids, peptides, and proteins; however, due to its high reactivity, it also tends to slowly react with water over time, thus hampering its long-term bioconjugation ability.30–32 By combining 1H-NMR and diffusion ordered spectroscopy experiments, Zangoli et al demonstrated that NPs-NHS are stable in water and hydrolysis, to release free N-hydroxysuccinimide, occurs slowly over several hours.41 XPS is another very sensitive technique that allows a detailed investigation of NPs’ shell enabling the determination of (a) elemental ratio and (b) bond state of the elements.76 XPS analysis was fundamental to confirm the generation of an oxidized shell on P3HT-NPs, through a postfunctionalization treatment, thus proving the formation of the first example of core@shell PT-NPs.38 To get further insights into the structural organization of PT within the NPs, X-ray diffraction (XRD) or high-resolution TEM (HR-TEM) measurements are extremely useful, despite requiring a throughout drying of the sample before their analysis. XRD and HR-TEM allow obtaining information on the crystalline structure, nature of the phase, lattice parameters, and crystalline grain size.69,76 For example, grazing-incidence wide-angle X-ray scattering experiments performed on P3HT-NPs of different sizes have highlighted the coexistence in the nanostructure of both large crystalline domains, randomly oriented independently of the size and amorphous regions.39 Nevertheless, the size of the crystalline domains, calculated by using the Scherrer’s formula,54,83 is closely related to the NPs size: the larger the NPs size, the larger the crystalline domain.39

Knowing the optical properties of NPs is of great relevance for their application in photobiology and photobiochemistry. Absorption and photoluminescence (PL) spectroscopies allow to study and characterize the absorption and emission of PT-NPs, the nature of the elementary photoexcitations, and their dynamics in the NPs in different media relevant to biology, as well as to evaluate the stability of the suspension.84 Furthermore, from the optical properties, it is possible to achieve information on NPs size, shape, concentration, aggregation state, and surface modification. It is interesting to note that differently from the emission the absorption spectra of PT-NPs are dependent on their size. More in general, the smaller NPs show blue-shifted structureless spectra while the bigger ones exhibit well-structured and strong red-shifted spectra.50,51,85,86

Finally, to get information on crucial parameters such as highest occupied molecular orbital/lowest unoccupied molecular orbital energy levels, oxidation/reduction potentials and ionization potential/electron affinity, cyclic voltammetry on PT-NPs can be performed.38

As shown the use of different techniques is often required to get a full picture of the characteristics of the nanostructure but to also evaluate well and completely even a single property.

4 | BIOLOGICAL APPLICATIONS OF PT-NPs

The interest in semiconducting PT-NPs for biological applications has gained growing attention as they show high brightness, good stability toward light, heat and oxygen, large absorption cross-sections, functionalizable surface, and attractive optical properties.15 Furthermore, their good biocompatibility and easy water dispersibility make them very appealing systems for in vivo biological applications. Thanks to the characteristics mentioned above, in the last decade a steadily increasing number of publications concerning PT-NPs applications as potential light nanotrasducers, that is, interfaces that convert light into a bioelectrical/biochemical signal, have been reported30–34,87–90 PT-NPs may operate as camouflage biocompatible interfaces—as they are close to the size of biological molecules and made up of biocompatible elements, such as carbon and sulfur—able to localize the signal transduction on their surface, thus eliciting a response in live cells/neurons/animals. Owing to the wide range of possible applications, herein, we will focus on some example relating to their use as light-activated bioactuators, that is, exogenous components that bestow light sensitivity to living matter and empower light control of physiological functions with a high degree of spatiotemporal resolution. Besides, some examples of PT-NPs applied as agents for photoacoustic imaging (PAI), photothermal (PTT), and photodynamic (PDT) therapies of tumors will be described.

4.1 | Functionalized PT-NPs as light-activated bioactuators

PT-NPs are currently under investigation for their potential use as noninvasive and highly specific light-activated bioactuators, that is, optical biointerfaces capable of triggering cellular activity upon photoexcitation, to repair/restore lost or damaged sensory functions.30,49,91 Similar to natural photoreceptors, PT-NPs can transduce the primary stimulus of light into a localized secondary stimulus that leads to a biochemical response. The phototransduction mechanism, which occurs at the abiotic/biotic interface between the cell and NPs, is still a matter of debate; however, three possible coupling mechanisms have been proposed.30 They refer to thermal,
electrical, and photochemical effects, even if the latter two seem to be the most accredited ones. In particular, P3HT-NPs have been shown to behave as light-sensitive interfaces for retinal neurons, both in vitro and in vivo. Lanzani and Benfenati’s groups, in previous papers, had demonstrated that P3HT, deposited as an active semiconductor layer in planar interfaces (2D), can be employed as a biocompatible material for neuronal photostimulation in retinal prostheses. However, the shortcomings presented by 2D prosthetic approaches—that are (a) low spatial resolution compared to that of foveal cones, (b) the requirement of invasive surgical implants, and (c) inflammatory reactions in the surrounding tissue upon device implantation—pushed toward the exploration of multifunctional nanostructured 0D materials as an innovative approach to create “injectable,” noninvasive, and highly specific light-activated bioactuators for in vitro/in vivo neural stimulation.

4.1.1 In vitro applications

Zucchetti et al reported the first assessment of P3HT-NPs biocompatibility in living cells, specifically HEK-293 cells. P3HT-NPs, prepared in sterile conditions to avoid microbial contamination, are efficiently internalized within live cells by endocytosis and distributed in the cytosol (as confirmed by laser scanning confocal microscopy) (Figures 5A and 6A). They exhibit excellent biocompatibility and do not alter the physiological functions of live cells, as proved by electrophysiology and calcium imaging experiments. Furthermore, the morphology and photophysical properties of NPs are preserved within the cellular environment, as shown by SEM analysis and in situ time-resolved PL (Figure 5A-C). In a later paper, Bossio et al investigated the photoinduced coupling mechanism between P3HT-NPs and HEK-293 cells and unveiled that, upon illumination, the presence...
of NPs leads to an increase in the intracellular production of reactive oxygen species (ROS)—such as singlet oxygen (\(^{1}\text{O}_2\)), free hydroxyl radicals (OH\(^{•}\)), hydrogen peroxide (H\(_2\)O\(_2\))—which in turn modulates Ca\(^{2+}\) dynamics without affecting cell viability (Figure 5D-F). Based on these results, the authors hypothesized that the observed changes in ROS concentration and Ca\(^{2+}\) dynamics are caused by a direct photocatalytic effect, thus ruling out thermal effects as the primary consequence of photoexcitation. The optical excitation of the NPs, under continuous wave light, mainly generates polaron charged states on their surface, which can react with the oxygen present in the environment, leading to the formation of ROS.

It is important to underline that ROS at low concentrations can regulate different biologic and physiological processes, while their overproduction is highly harmful to the cells since they induce nonspecific damage of proteins, lipids, and DNA. For this purpose, the authors, through a series of dose-response analysis and cell viability experiments, were able to identify the most suitable P3HT-NPs concentration (corresponding to 0.2 optical density value of the colloidal dispersion) to achieve an optimal functional photomodulation of ROS generation without causing cytophototoxicity effects.

As shown, the absence of specific targeting groups on the surface of P3HT-NPs does not allow regulating parameters such as the efficiency of cell loading, colocalization, biodistribution, and so on. However, thanks to the versatility of thiophene chemistry, functionalized NPs have been synthesized and investigated in recent years. For instance, NPs decorated with NHS reactive groups on their surface, which can react with the oxygen present in the environment, leading to the formation of ROS.

The optical excitation of the NPs, under continuous wave light, mainly generates polaron charged states on their surface, which can react with the oxygen present in the environment, leading to the formation of ROS.

4.1.2 | In vivo applications

PT-NPs have been proved to induce light sensitivity in living organisms. Tortiglione et al tested P3HT-NPs as light-activated bioactuators in freshwater polyp Hydra vulgaris, a small animal used as a model organism because of its eyeless nature.

As shown, the absence of specific targeting groups on the surface of P3HT-NPs does not allow regulating parameters such as the efficiency of cell loading, colocalization, biodistribution, and so on. However, thanks to the versatility of thiophene chemistry, functionalized NPs have been synthesized and investigated in recent years. For instance, NPs decorated with NHS reactive groups on their surface can dock to the cellular membrane of live HEK-293 cells, probably because of a reaction between the NHS groups present on the NPs surface and the primary amines of membrane proteins (Figure 6B). These NPs, however, resulted to be less biocompatible than P3HT-NPs, suggesting that further refinement of the NPs structure is needed to prevent cytotoxicity. Despite this, they are able to induce a significant biphasic change in the membrane potential of the cells upon illumination, that is, depolarization followed by hyperpolarization, highlighting the crucial role of bioconjugation in boosting phototransduction effects.

It has also been reported that it is possible to prepare chiral PT-NPs by functionalizing the side chains of polymers with enantiopure R or S chiral moieties. Chiral nanoobjects may interact differently with chiral biomolecules and affect physiological events. Both R and S PT-NPs are efficiently internalized by NIH-3T3 fibroblast cells, via clathrin-mediated endocytosis mechanisms, and are not cytotoxic. However, the different chirality of the NPs induces a different level of protein adsorption on their surface, the so-called protein “corona.” This enables cells to discriminate between enantiomeric R and S NPs. Indeed, a difference in cellular uptake and intracellular localization has been observed in such systems, resulting in the first example of enantioselectively in the biological interaction between PT-based nanomaterials and living systems (Figure 6C).

4.1.2 | In vivo applications

PT-NPs have been proved to induce light sensitivity in living organisms. Tortiglione et al tested P3HT-NPs as light-activated bioactuators in freshwater polyp Hydra vulgaris, a small animal used as a model organism because of its eyeless nature.
procedure, they are distributed throughout the subretinal space without causing trophic effects on the residual photoreceptors or significant inflammatory reactions (Figure 7A,B). Remarkably, NPs bestow inner retinal neurons with light sensitivity (Figure 7C,D). Indeed, blind rats completely regain subcortical visual responses, visual acuity, and light-driven behaviors at levels indistinguishable from those of healthy congeneric rats for up to 8 months after a single injection (Figure 7G,H). Notably, over time, P3HT-NPs do not show any tendency to migrate toward the inner retinal layers and maintain the original subretinal distribution, thus excluding a wide-body diffusion of the injected NPs or their clearance by neurons or glia (Figure 7E,F). The results obtained by employing P3HT-NPs are equivalent to the best achieved with current 2D implants. Therefore, they represent a promising approach for the treatment of degenerative retinal diseases because of low-invasive surgery, the permanence of the effects, and the potential for a high spatial resolution.

4.2 PT-NPs for photo-activated therapies

Over the past decade, photo-activated therapies, such as PDT and PTT, have seen a large research interest as they promise the development of efficient and noninvasive cancer therapies capable of destroying, with high specificity and efficacy, tumoral tissue with minimal side effects. In this fast-growing field, organic semiconducting NPs and, in particular, PT-NPs, are employed as innovative noninvasive light-transducing nanoagents because of their multiple properties such as suitable size, shape, functionalization, dispersibility in an aqueous medium, good biocompatibility, excellent photostability, and large extinction coefficient. Besides, the great structural versatility of the thiophene ring allows synthesizing numerous thiophene-based chemical structures enabling the preparation of NPs whose light absorption can be finely tuned ranging from UV-Vis (400-700 nm) to NIR (750-1700). The use of NIR light in biological environments is strongly recommended because the absorption of photons in this region leads to minimal photodamage and presents an increased penetration depth in tissue, allowing the photoactivation of PT-NPs in living subject. Moreover, in this region, the absorption by tissue components, that is, hemoglobin, water and lipid, is drastically reduced as they mainly absorb in the UV-Vis spectral range. According to this, research has been directed toward the synthesis of new NPs capable of absorbing light in the NIR region of the spectrum. Furthermore, several articles published by different research
groups have demonstrated the possibility of synthesizing PT-NPs with optical properties covering not only the first NIR-I, 750-1000 nm, but also the second region extending from 1000 to 1700 nm (NIR-II). Concerning the mechanism of action, in general, under illumination, PT-NPs act as light nanotransducers. In PDT, they efficiently transfer light energy to oxygen molecules forming through different photochemical reactions ROS, including free OH• and 1O2, that are highly cytotoxic for cells (Figure 8). In PTT, differently, PT-NPs convert light energy into localized heat, which causes hyperthermia in the local environment surrounding the NPs, leading to irreversible damage to cancer cells and tissues, which is locally exposed to light (Figure 8).

Notably, the absorption of NIR light by PT-NPs can also generate ultrasonic waves that, if received by acoustic detectors, produce high-resolution images (mm) of deep tumors (cm). This makes PT-NPs intrinsic contrast agents for PAI, that is, an emerging imaging technology with potential for preclinical biomedical research and clinical applications.

### 4.2.1 PDT

For PDT applications, some key characteristics have to be installed in PT-NPs such as (a) absorption within the therapeutic window (i.e., 700-1000 nm), (b) efficient ROS generation, (c) negligible dark toxicity and side effects, (d) water dispersibility, (e) good membrane affinity, and (f) photostability. PT-NPs can be activated via visible or NIR light. However, visible light (<700) has the disadvantage that it cannot penetrate deeply into the tissue, because of absorption by tissue components, and this limits the application of NPs activated by visible light to superficial tumor identification and therapy because of the high risk of photodamage to healthy tissues. Otherwise, the NIR region, where biological damage and scattering are smaller than in the ultraviolet-visible (UV-Vis), allows better tissue penetration and a high spatial localization. Thus, lately, different classes of NIR light excited NPs have been developed. Recently, two-photon excitation (TPE) has been proved to be a viable technique to selectively excite NPs with NIR light. TPE refers to the nonlinear absorption of two low-energy NIR light photons simultaneously that promote a molecule to the excited state corresponding to the combined energy of the two-photons. Hence, the resulting high-energy excited state can sensitize oxygen to generate cytotoxic ROS, which in turn can kill cancer cells. Guo et al prepared very small NPs (10-15 nm), based on a polythiophene quaternary ammonium salt, that are able to spontaneously cross the cellular membrane of HeLa cells and distribute specifically into lysosomes. These NPs, whose maximum of absorption is around ~410 nm, can efficiently generate 1O2 both by one-photon (532 nm laser irradiation) and by two-photon absorption (800 fs laser). In vitro and in vivo experiments demonstrated that in presence of NPs, upon 800 nm fs pulse laser excitation, it is possible to achieve deep-tissue imaging of the mock tissue (up to 2100 µm) and highly efficient in vivo PDT of cancerous tissue.
An alternative strategy to promote NPs’ adsorption in the NIR is to intervene on the chemical structure of the polymer. The design of push-pull PTs alternating electron-donating (D) and electron-acceptor (A) moieties into their structure is a powerful strategy to control the optoelectronic properties of materials and extend their absorption in the NIR region. Zhou et al engineered a PT by introducing thiophene units as D moieties and benzo thiadiazole/diketopyrrolopyrrole as A units to push the absorption toward NIR-I. Furthermore, cationic groups were also added to the side chains to facilitate the interaction with the negatively charged membrane of the tumor cells. The corresponding NPs, which present absorption in the same range of the “therapeutic window,” under irradiation at 808 nm efficiently generate ROS. In addition, they are also able to convert some of the adsorbed light into heat, thus obtaining a superior therapeutic effect due to a synergistic treatment. Indeed, in vitro and in vivo experiments demonstrated that these NPs can kill cancer HeLa cells and inhibit tumor growth in mice.

Although PDT has great promises for cancer therapy, its efficacy is, however, often compromised by tumor hypoxia, that is, the scarce amount of oxygen present in the cellular environment that limits its therapeutic effect. A viable strategy to overcome this issue is to combine PDT with chemotherapy. For instance, Cui et al reported the synthesis of a semiconducting push-pull PT-based nanoprodrug functionalized with a chemotherapeutic drug, that is, a bromoisophosphoramidemustard intermediate (IPM-Br) able to induce DNA cross-linking and cellular apoptosis. The NPs synthesized from this material, under irradiation at 808 nm, are capable of efficiently generating \( ^1 \text{O}_2 \) and, at the same time, the hypoxic tumor microenvironment activates the chemotherapeutic action, through the fragmentation and release of IPM-Br catalyzed by nitroreductase that leads to cell death.

Another technique to increase the therapeutic effect of PDT in tumor ablation is the one recently reported by Li et al, wherein PT-NPs have been functionalized with an NIR photoactivatable function containing an aminonucleoside, that is, a puromycin able to inhibit intracellular protein synthesis and induce cell death. NIR irradiation of the NPs allows the on-demand production of \( ^1 \text{O}_2 \), which, in addition to killing tumor cells,
triggers the breakage of the $^{1}\text{O}_2$-cleavable linker by locally releasing caged puromycin. Notably, compared to sole PDT effect, this synergistic action leads to a 2.2-fold higher cell killing capacity and significantly reduced tumor growth in a mouse model. The same authors reported the synthesis of organic semiconducting pro-nanoenzyme (OSPE) and pro-nanostimulant (OSPS), that is, PT-NPs conjugated with an inactive proenzyme (EBAP) or an immunostimulant (NLG919) via a $^{1}\text{O}_2$-cleavable linker, to exploit a photoactivated synergistic therapeutic action. In both cases, NIR irradiation enabled the generation of cytotoxic $^{1}\text{O}_2$. Furthermore, in the presence of OSPE the release of the proenzyme triggers a spontaneous cascade reaction leading to the degradation of intracellular ribonucleic acid (RNA) thus inducing cell death. On the other hand, in the case of OSPS, the release of the inhibitor NLG919 blocks the function of a specific immune-suppressive enzyme (IDO), overexpressed by most cancer cells, which catalyzes the degradation of the essential amino acid tryptophan (IDO), overexpressed by most cancer cells, which catalyzes the degradation of the essential amino acid tryptophan, thus inducing cell death. In 4T1 tumors, and their potential use in biomedical applications and clinical practices. An ideal PTT agent should have: (a) low toxicity, (b) high photothermal conversion efficiency (photon energy $\rightarrow$ heat) (PTE), (c) efficient accumulation in tumors, and (d) easy and reproducible synthesis.

Push-pull PT-based NPs possessing absorbance in the NIR-I optical window (700-900 nm) have been widely explored by many research groups to selectively ablate tumors with minimal invasiveness and low toxicity to normal tissues. For example, Li et al reported the preparation of small-sized NPs (~30 nm) using polymers containing diketopyrrolopyrrole (A) bridged with different thiophene units (D), which exhibit NIR absorption between 600 nm and 900 nm. NPs, upon irradiation at 808 nm (0.5 W cm$^{-2}$), showed a high PTE (up to 65%) and high photostability (five heating/cooling cycles), thus leading to the ablation of 4T1 tumor cells both in vitro and in vivo.

More recently, NPs excitable by light in the NIR-II window (1000-1700 nm) have aroused even more interest, given the possibility of employing radiation with a longer wavelength that promise to improve: (a) penetration depth (~10 mm), (b) spatial resolution (~3 μm), (c) signal-to-noise ratio (up to ~15), and (d) the maximum permissible exposure to laser (the power safety limit for 1064 nm is three-fold higher than that of 808 nm laser).

Wei et al prepared NPs, based on a diketopyrrolopyrrole/thiophene polymer, with a size around 200 nm and absorption reaching 1100 nm. The shift in the absorption wavelength toward the NIR-II window allowed the investigation of their photothermal properties by using a laser at 1064 (1 W cm$^{-2}$) instead of conventional 808 nm lasers. Thanks to their excellent PTE (49.5%) under NIR-II laser irradiation, NPs presented outstanding anticancer ability both in vitro (HeLa cells) and in vivo (on tumor xenografts in nude mice) already after few minutes of irradiation. Furthermore, their PTT ability was demonstrated to be strongly concentration dependent, indeed the increase in temperature is directly related to an increase in NPs concentration.

As shown, the use of NIR II allows performing PTT under safe laser energy; however, the effective NIR-II brightness is relatively low for biological imaging, thus limiting their applicability in preclinical research and clinical practice. Yang et al reported that the synthesis of polymers in which the D moieties present an extended thiophene-based fused ring system allows forming NPs (~50 nm) with high molar extinction coefficients and longer excitation wavelengths (Figure 10A). The synthesized NPs present quantum yield in the NIR-II region of 1.25% and PTE of 38% thus entailing their use for both NIR-II imaging and PTT under safe laser energy. The high brightness allowed for real-time visualization of both the whole body of healthy mice - imaging small capillaries (198 μm) by using ultralow illumination power (0.25 W cm$^{-2}$) and short exposure time (30 ms) - and tiny brain vessels (~1.9 μm) with high clarity (Figure 10A,B). In addition, the detection of cerebral ischemic stroke and tumors, which is crucial to accurately guide therapy and minimize injury to the surrounding healthy tissue, was also possible. The PTT efficacy of these NPs has been demonstrated in vivo on 4T1 tumor bearing mice, finding that 8 minutes of excitation with a 980 nm laser (0.72 W cm$^{-2}$) is sufficient to kill tumor cells.

It is important to note that PTT NPs are also intrinsic contrast agents for PAI, thus simultaneously allowing therapy and imaging. PAI - which combines optical excitation with ultrasonic detection - compared to traditional optical imaging techniques (e.g., fluorescence) provides deeper tissue imaging penetration with higher spatial resolution, thanks to the fact that the scattering of ultrasonic signals is much weaker than that of optical signals in biological tissues.
cooling cycles). Furthermore, a remarkable PTT effect under 1064 nm laser irradiation (0.90 W cm\(^{-2}\)) both \textit{in vitro} toward HeLa and HepG2 cells and \textit{in vivo} on tumor bearing nude mice was found. Notably, NPs result to be excellent PAI agents with strong photoacoustic signals useful for imaging-guided cancer therapy. It is important to highlight the relation between PA signal and concentration: the higher PA signal, the higher NPs concentration. Guo et al demonstrated that PT-based NPs (~60 nm) decorated with cyclo(Arg-Gly-Asp-DPhe-Lys[mpa]) — a targeting ligand able to bioconjugate αVβ3 integrin receptors overexpressed in endothelial cells of the brain tumor as well as on glioblastoma cells — can be employed for precise PAI and spatiotemporal PTT of brain tumor via scalp and skull (Figure 10B).\(^{47}\) NPs functionalization significantly enhances tumor cell uptake, thus increasing PA signals of 3.5-fold compared to the corresponding NPs without decoration. Through a real-time PAI system, NPs help to identify glioma at a depth of ~3 mm via scalp and skull with a high signal-to-background ratio (90), and thanks to their PTT properties (PTE 30.1%), upon treatment at 1064 nm (1 W cm\(^{-2}\)), the tumor progression is effectively constrained. Therefore, NIR-II semiconducting PT-based NPs provide a multifunctional nanoplatform that results promising for the treatment of brain tumors.

Furthermore, Jiang et al demonstrated that NIR II PT-NPs, consisting of alternating thiophene functionalized units and benzobistadiazole moieties, can be completely metabolized and excreted after administration to living mice, a fundamental requirement for any clinical application.\(^{166}\)

5 | SUMMARY AND OUTLOOK

This review summarizes the latest developments in the synthesis and characterization of semiconducting PT-NPs. Nowadays, thiophene-based polymers, owing to efficient and robust synthetic methodologies, can be prepared "à la carte" by exploiting the facile functionalization of the thiophene ring. Through nanoprecipitation and miniemulsion methods almost all TpM can be easily organized into water dispersed PT-NPs. These nanomaterials have been proved to present tunable optical properties, excellent photostability, strong light-harvesting ability, low cytotoxicity, and high brightness. These properties have already enabled their successful applications in biomedical imaging and have promoted their implementation as light-transducing nanoagents for the cure of retinal degenerative diseases. This review aims at highlighting the high level of structural sophistication reached by chemists in preparing novel
PT-NPs and the great untapped potential that they have in biology and medicine, to foster the multidisciplinary approach required to unveil new and unprecedented applications for this intriguing family of nanomaterials.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGMENT
The authors acknowledge the UE project INFUSION (Engineering optoelectronic Interfaces: a global action intersecting Fundamental concepts and technology implementation of self-organized organic materials; proposal number: 734834).

ORCID
Francesca Di Maria https://orcid.org/0000-0001-5557-3816

REFERENCES
1. I. F. Perepichka, D. F. Perepichka, *Handbook of Oligo- and Polythiophenes: Applications in Organic Electronics and Photonics*, 1st ed., John Wiley & Sons Inc, Hoboken 2009.
2. D. Fichou, *Handbook of Oligo- and Polythiophenes*, Wiley-VCH, New York 2008.
3. M. L. Capobianco, G. Barbarella, A. Manetto, *Molecules* 2012, 17, 910.
4. P. Sista, K. Ghosh, J. S. Martinez, R. C. Rocha, J. Nanosci. Nanotechnol. 2014, 14, 250.
5. S. C. Rasmussen, S. J. Evenson, C. B. McCausland, *Chem. Commun.* 2015, 51, 4528.
6. U. Mehmoon, A. Al-Ahmed, I. A. Hussein, *Renew. Sustain. Energy Rev.* 2016, 57, 550.
7. T. P. Kaloni, P. K. Giesbrecht, G. Schreckenbach, M. S. Freund, *Chem. Mater.* 2017, 29, 10248.
8. E. Salatelli, M. Marinelli, M. Lanzì, A. Zanelì, S. Dèll’Elce, A. Liscio, M. Gazzano, F. Di Maria, *J. Phys. Chem. C* 2018, 122, 4156.
9. F. Lodola, V. Rosti, G. Tullii, A. Desì, L. Tapella, P. Catarsi, D. Lim, F. Moccì, M. R. Antognazza, *Sci. Adv.* 2019, 5, eaav4620.
10. G. Barbarella, M. Melucci, G. Sotgiu, *Adv. Mater.* 2005, 17, 1581.
11. F. Di Maria, P. Olivelli, M. Gazzano, A. Zanelì, M. Biasiucci, G. Gigli, D. Gentili, P. D’Angelo, M. Cavallì, G. Barbarella, *J. Am. Chem. Soc.* 2011, 133, 8654.
12. I. Viola, I. E. Palamà, A. M. L. Coluccia, M. Biasiucci, B. Dozza, E. Lucarelli, F. Di Maria, G. Barbarella, G. Gigli, *Integr. Biol. (United Kingdom)* 2013, 5, 1057.
13. I. E. Palamà, F. Di Maria, S. D’Amone, G. Barbarella, G. Gigli, *J. Mater. Chem. B* 2015, 3, 151.
14. F. Di Maria, G. Barbarella, *J. Sulfur Chem.* 2013, 34, 627.
15. G. Barbarella, M. Zangoli, F. Di Maria, *Adv. Heterocycl. Chem.* 2017, 123, 105.
16. F. Di Maria, M. Zangoli, I. E. Palamà, E. Fabiano, A. Zanelì, M. Monari, A. Perinot, M. Caironi, V. Maiorano, A. Maggiore, M. Pugliese, E. Salatelli, G. Gigli, I. Viola, G. Barbarella, *Adv. Funct. Mater.* 2016, 26, 6970.
17. S. Ellinger, K. R. Graham, P. Shi, R. T. Farley, T. T. Steckler, R. N. Brookins, P. Taranekar, J. Mei, L. A. Padilha, T. R. Enseny, H. Hu, S. Webster, D. J. Hagan, E. W. Van Stryland, K. S. Schanje, J. R. Reynolds, *Chem. Mater.* 2011, 23, 3805.
18. S. Canola, L. Mardegan, G. Bergamini, M. Villa, A. Accocella, M. Zangoli, L. Ravotto, S. A. Vinogradov, F. Di Maria, P. Ceroni, F. Negri, *Photochem. Photobiol. Sci.* 2019, 18, 2180.
19. M. Marinelli, M. Lanzì, A. Liscio, A. Zanelì, M. Zangoli, F. Di Maria, E. Salatelli, *J. Mater. Chem. C* 2020, 8, 4124.
20. H. Sun, X. Guo, A. Facchetti, *Chem. 2020*, 6, 1310.
21. F. Di Maria, E. Fabiano, D. Gentili, M. Biasiucci, T. Salzillo, G. Bergamini, M. Gazzano, A. Zanelì, A. Brillante, M. Cavallì, F. Delta Sala, G. Gigli, G. Barbarella, *Adv. Funct. Mater.* 2014, 24, 4943.
22. F. Di Maria, M. Zangoli, M. Gazzano, E. Fabiano, D. Gentili, A. Zanelì, A. Fermi, G. Bergamini, D. Bonifazi, A. Perinot, M. Caironi, R. Mazzaro, V. Morandi, G. Gigli, A. Liscio, G. Barbarella, *Adv. Funct. Mater.* 2018, 28, 1801946.
23. M. Zangoli, M. Gazzano, F. Montì, L. Mainì, D. Gentili, A. Liscio, A. Zanelì, E. Salatelli, G. Gigli, M. Baroncini, F. Di Maria, *ACS Appl. Mater. Interfaces* 2019, 11, 16864.
24. M. Zangoli, F. Di Maria, G. Barbarella, *ChemistryOpen* 2020, 9, 499.
25. J. E. Millstone, D. F. J. Kavulak, C. H. Woo, T. W. Holcombe, E. J. Westling, A. L. Brisenò, M. F. Toney, J. M. J. Fréchet, *Langmuir* 2010, 26, 13056.
26. F. Di Maria, I. E. Palamà, M. Baroncini, A. Barbieri, A. Bongini, R. Bizzarri, G. Gigli, G. Barbarella, *Org. Biomol. Chem.* 2014, 12, 1603.
27. G. Barbarella, F. Di Maria, *Acc. Chem. Res.* 2015, 48, 2230.
28. M. Moros, F. Di Maria, P. Dardano, G. Tommasini, H. Castillog-Michel, A. Kostun, M. Zangoli, M. Blassio, L. De Stefano, A. Tino, G. Barbarella, *C. Tortiglione, iScience* 2020, 23, 101022.
29. P. Koralli, A. D. Nega, L. E. Vagiaki, A. Pavlou, M. G. Siskos, A. Dimitrakopoulou-Strauss, V. G. Gregoriou, C. L. Chochos, *Mater. Chem. Front.* 2020, 4, 2357.
30. F. Di Maria, F. Lodola, E. Zucchetti, F. Benfenati, G. Lanzani, *Chem. Soc. Rev.* 2018, 47, 4757.
31. Y. Jiang, K. Pu, *Acc. Chem. Res.* 2018, 51, 1840.
32. Y. Wang, L. Feng, S. Wang, *Adv. Funct. Mater.* 2019, 29, 1806818.
33. S. N. Clafton, D. M. Huang, W. R. Massey, T. W. Kee, A. Dimitrakopoulou-Strauss, V. Gregoriou, C. L. Chochos, *Mater. Chem. Front.* 2013, 117, 4626.
34. S. Cifci, A. J. C. Kuehne, *In Direct Synthesis of Conjugated Polymer Nanoparticles* (Eds: B. Liu), Wiley-VCH, Weinheim, Germany, 2018, 35.
35. J. Pecher, S. Mecking, *Chem. Rev.* 2010, 110, 6260.
36. L. Feng, C. Zhu, H. Yuan, L. Liu, F. Lv, S. Wang, *Chem. Soc. Rev.* 2013, 42, 6620.
37. H. Shimizu, M. Yamada, R. Wada, M. Okabe, *Polym. J.* 2008, 40, 33.
38. F. Di Maria, A. Zanelì, A. Liscio, A. Kostun, E. Salatelli, R. Mazzaro, V. Morandi, G. Bergamini, A. Shaffer, S. Rozen, *ACS Nano* 2017, 11, 1991.
39. T. Moreira, C. A. T. Laia, M. Zangoli, M. Antunes, F. Di Maria, S. De Monte, F. Liscio, A. J. Parola, G. Barbarella, *ACS Appl. Polym. Mater.* 2020, 2, 3301.
AUTHOR BIOGRAPHIES

Mattia Zangoli graduated from the University of Bologna (Italy) and received his Ph.D. in 2018 from the University of Bologna (Italy). He is currently a researcher at the Institute of Organic Synthesis and Photoreactivity (ISOF) of the Italian National Research Council (ISOF-CNR). His research interests are focused on the synthesis of oligo- and polythiophens and their organization into 0D and 1D structures.

Francesca Di Maria graduated from the University of Catania (Italy) and received her Ph.D. in 2016 from the University of Bologna (Italy). She is currently a researcher at the Institute of Organic Synthesis and Photoreactivity (ISOF) of the Italian National Research Council (ISOF-CNR). Her main research activity deals with the synthesis and characterization of thiophene-based materials and their organization into supra-molecular nano- and microstructures (0D and 1D) for application in optoelectronics, photonics, and biological systems. In this field, she has coauthored over 40 scientific publications and two book chapters.

How to cite this article: Zangoli M, Di Maria F. Synthesis, characterization, and biological applications of semiconducting polythiophene-based nanoparticles. VIEW. 2021;2:20200086.
https://doi.org/10.1002/VIW.20200086