**Light-triggerable formulations for the intracellular controlled release of biomolecules**

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**Abstract (Max: 100 words)**

New therapies based on the use of biomolecules (e.g. proteins, peptides, non-coding RNAs) have emerged during the last years. Due to their instability, side effects, and limitations to cross cell membrane, delivery systems are required to fully show their biological potential. Sophisticated nanoformulations responsive to light offer an excellent opportunity for the controlled release of these biomolecules, enabling the control of timing, duration, location and their dosage. This review discusses design principles for the delivery of biomolecules, in particular proteins and RNA-based therapeutics, by light-triggerable formulations. It further discusses opportunities offer by these formulations in terms of endosomal escape as well as their limitations.

**Teaser (25-30 words):**

Increasing numbers of proteins and non-coding RNAs are being described as new therapeutic agents. The controlled release of these biomolecules may offer opportunities in the context of several biomedical applications.

**Research highlights (3 to 5 bullet points):**

* Increasing number of biomolecule-based therapies approved
* Light-responsive nanoformulations enable spatio-temporal control
* Laser irradiation can promote endosomal escape of nanocarriers and cargos
* Laser irradiation of nanoformulations already approved for photodynamic therapy

1. **Introduction**

Advances in the cell biology field have opened the way to a new collection of therapeutic agents comprised by biomolecules, such as proteins and non-coding RNAs, namely siRNAs and microRNAs (miRNAs) [[1](#_ENREF_1),[2](#_ENREF_2)]. There are more than 130 different proteins or peptides approved for clinical use by the US Food and Drug Administration (FDA), and many more are in development [[2](#_ENREF_2)]. Although some of these proteins do not require internalization to exert a biological effect since they target cell membrane receptors, others do require internalization to trigger a biological response. Indeed, the intracellular delivery of several proteins, such as superoxide dismutase, caspase 3, ribonuclease A has already demonstrated potential to treat different diseases, like neuro-cardiovascular diseases [[3](#_ENREF_3)] and cancer [[4](#_ENREF_4)]. There is also a battery of transcription factors for direct cellular reprogramming that have not reached the clinic [[5](#_ENREF_5)]. Several therapies based in non-coding RNAs (e.g. siRNAs, miRNAs, mRNA) have also emerged in the last years [[6](#_ENREF_6),[7](#_ENREF_7)]. In the last 5 years, FDA has approved 3 RNA-based therapies and many others are being evaluated in pre-clinical programs [[1](#_ENREF_1)].

Due to the susceptibility of these biomolecules to degradation and instability in serum, appropriate delivery systems must be designed. Albeit most delivery systems already approved or in clinical trials are based on passive drug release [[8](#_ENREF_8)], there has been intensive research on the development of stimuli-responsive nanoformulations [[9](#_ENREF_9)] for controlled delivery of biomolecules. The relevance of such strategies is driven by the possibility of reaching different levels of control: i) timing; ii) duration; iii) location iv) dosage and v) independent release of multiple drugs. These nanoformulations may optimize drug efficacy while minimizing side effects. Therefore, new methods have been developed to control drug delivery either by the susceptibility of the nanocarrier to changes in its microenvironment or by an external stimulus triggered by an operator. Accordingly, stimuli-responsive nanocarriers may respond to endogenous stimuli such as pH, enzymatic activity, redox environment/glutathione concentration, among others [[9](#_ENREF_9)]. A controlled drug delivery may also be achieved using magnetic, ultrasound, thermo and light sensitive materials that respond to an external stimulus [[10](#_ENREF_10),[11](#_ENREF_11)]. The remote activation of the nanocarrier may enable repeated and reproducible dosing of the drug, reducing risks associated with toxicity. When compared to other external triggers, light offers some advantages: i) precise control of the size of the area being irradiated by varying the beam of the laser, providing a higher level of spatial control and ii) the possibility of having a system that responds to different wavelengths for multiple drug release.

In this short-review we summarize recent advances in the use of light-triggerable nanoformulations for the delivery of biomolecules, namely proteins/peptides and RNA-based therapeutics. We describe the synthetic strategies and design principles for its preparation. We then present several pre-clinical examples in the application of the formulations for the delivery of biomolecule-based therapeutics, followed by a discussion about their advantages and limitations.

### The chemistry behind light-triggerable release systems

Although no light-triggerable drug delivery system has yet been translated into clinical trials [[12](#_ENREF_12)], light has already been used in a clinical setting to treat different diseases [[13](#_ENREF_13)]. Light-triggerable systems can respond to ultraviolet (UV), visible or near infrared (NIR) light depending on the type of materials and light-responsive mechanisms associated. A considerable part of the light-triggerable systems is constituted by compounds that respond only to UV light, since it is a high-energy radiation capable of inducing conformational changes or break covalent bonds [[12](#_ENREF_12),[14](#_ENREF_14)]. However, a major drawback associated to UV light as an external stimulus is the low penetration depth, narrowing potential biomedical applications to organs easily accessible, such as the skin and the eye.

In order to overcome the disadvantages associated to UV light, new systems using nanocarriers responsive to NIR irradiation have been developed. The main advantage of NIR irradiation (650-900 nm) is the low absorbance by skin and tissue, enabling a penetration depth on the order of hundreds of micrometres to centimetres [[15](#_ENREF_15)]. The energy of the NIR irradiation may be enhanced for certain applications (e.g. photo cleavage applications, see below) by the use of two-photon technology. In this case, the NIR labile compound absorbs two photons in the NIR region [[16](#_ENREF_16),[17](#_ENREF_17)]. In general, the probability of excitation by the absorption of two photons is very low, therefore this process requires the use of pulsed lasers in order to provide a high density of photons in short pulses (nanosecond to femtosecond scale) and the use of photolabile groups with larger two photon cross-section (e.g. coumarin derivatives) [[18](#_ENREF_18)].

Light can interact directly with chromophores and induce changes in the nanocarriers through different mechanisms [[12](#_ENREF_12),[19-21](#_ENREF_19)]. These changes may be reversible or irreversible, direct or indirect. In case of direct changes the light interacts with a chromophore followed by: (i) the cleavage of a photolabile compound such as the ones based on nitrobenzyl or coumarin groups (photocleavage) [[22](#_ENREF_22)], (ii) the change in the conformation of the chromophore such as azobenzene or spiropyran (photoisomerization) [[23](#_ENREF_23)] and (iii) the change in the hydrophilic/hydrophobic ratio of molecules such as 2-diazo-1,2-naphthoquinone (photo-induced rearrangement) [[24](#_ENREF_24)].

Besides the direct interaction between light and chromophores, mechanisms of indirect photoactivation have been also tested. These include light upconversion, photothermal effect and photo-induced generation of radicals. In case of light upconversion, nanocarriers absorb NIR light followed by the conversion into visible or UV light, which in turn, leads to cleavage or isomerisation of release-triggerable compounds [[25](#_ENREF_25),[26](#_ENREF_26)]. In case of phototermal effect, the nanocarriers convert visible or NIR light into heat, which in turn, leads to conformational changes of thermo-sensitive materials immobilized in nanocarrier surface [[27](#_ENREF_27)] or the melting of nanocarrier surface [[28](#_ENREF_28)] leading, in both cases, to the release of drugs. In case of the photo-induced radical generation, the nanocarrier contains a photosensitizer that is able to generate radicals when they are irradiated with an appropriate wavelength. These radicals may then induce chemical changes in the material components, by attacking for example disulfide [[29](#_ENREF_29)] or double bonds [[30](#_ENREF_30)].

### 3. Light-triggerable nanocarriers for the delivery of biomolecules

**3.1 Design principles**

Different light-triggerable nanocarriers have been used for the delivery of biomolecules (**Tables 1 and 2**). Most of them are based on inorganic materials, namely plasmonic and upconversion nanocarriers, with few examples of organic formulations, such as polymeric micelles and nanogels. In the case of inorganic plasmonic formulations, the biomolecules are usually immobilized on the surface of the nanocarrier [[31](#_ENREF_31),[32](#_ENREF_32)]. The surface immobilization of the biomolecule has some limitations because it can hold in most cases a single monolayer of biomolecules and the internalization properties of the formulation are likely affected by the physico-chemical properties of the biomolecule. In case of inorganic upconversion formulations, a layer of mesoporous silica has been also explored for the encapsulation of the cargo [[33](#_ENREF_33),[34](#_ENREF_34)]. For light-triggerable polymeric nanocarriers, encapsulation of the cargo has been reported through the electrostatic interaction between the polymers and the biomolecules. Cleavage of light-sensitive moieties leads to imbalance of charges and nanocarrier disassembly with a concomitant release of the cargo [[35-37](#_ENREF_35)].

In case of RNA molecules, different approaches have been used for the surface nanocarrier immobilization including electrostatic immobilization in the surface [[38](#_ENREF_38),[39](#_ENREF_39)] as well as after their direct binding to the surface of the nanocarrier via thiol-gold chemistry [[40](#_ENREF_40)] (**Figure 1**). Regarding the first strategy, the cargo is released through photothermal destabilization of the interaction between RNA and the polymers covering the nanocarrier surface [[41](#_ENREF_41)]. Regarding the second strategy, the release of the cargo is achieved after the cleavage of thiol-gold bond induced by a femtosecond pulsed laser [[31](#_ENREF_31)].

The adsorption step of the nanocarrier to the cell membrane seems to be the rate limiting step in the internalization process [[42](#_ENREF_42)]. Therefore, the intracellular delivery of negatively charged biomolecules (e.g. proteins or siRNAs) immobilized on the nanocarrier surface may be hampered due to their negative charge and less likely interaction with the cell membrane. To overcome this issue, cell penetrating peptides [[31](#_ENREF_31)] or cationic polymers/coatings [[39](#_ENREF_39),[41](#_ENREF_41),[43](#_ENREF_43),[44](#_ENREF_44)] have been immobilized on the nanocarrier surface to enhance its uptake.

Below we describe some examples of light-activatable nanocarriers for the release of proteins/peptides and RNA-based therapeutics.

#### 3.2 Light-triggerable delivery of proteins/peptides

UV and NIR responsive nanoformulations have been described for the delivery of proteins/peptides (**Table 1**). In case the protein/peptide was not covalently conjugated to the nanocarrier there was some leaching (passive diffusion) of the biomolecule [[35](#_ENREF_35),[36](#_ENREF_36)]. The release kinetics were controlled by the chemistry of the formulation and ranged from a few seconds (<1 min) [[45](#_ENREF_45)] up to several hours (>10 h) [[35](#_ENREF_35),[36](#_ENREF_36),[46](#_ENREF_46)]. According to studies, the physico-chemical properties of the immobilized protein/peptide as well as the nanocarrier chemistry controlled the internalization rate of the formulation [[35](#_ENREF_35),[36](#_ENREF_36)]. Light-controlled intracellular delivery of proteins has been mainly restricted to proof-of-concept studies with fluorescent/model proteins, therefore the delivery of proteins for modulation of cell activity is yet to be validated [[46](#_ENREF_46)]. Gold nanocarriers have been described for the NIR light-induced release of transcription factors without inducing their degradation, albeit the effect of these proteins was not evaluated in cells [[32](#_ENREF_32)]. Similarly, gold nanocarriers have been described for the spatio-temporal delivery of pro-apoptotic peptides [[47](#_ENREF_47)]. An elegant strategy for the intracellular delivery of proteins is by the delivery of the protein production machinery (caged-DNA, RNA polymerase, and ribosomes) within the cell using a carrier [[38](#_ENREF_38)]. The production of the protein may be triggered by UV light activation. This concept has been demonstrated *in vitro* and *in vivo*. Overall, to the best of our knowledge, it remains elusive the use of light-triggerable protein/peptide delivery formulations to modulate cell activity. In addition, very few light-triggerable delivery systems for proteins/peptides have been tested *in vivo*.

#### 3.3 Light-triggerable delivery of RNA-based therapeutics

Nanoformulations controlled externally by light have been reported for the delivery of antisense oligonucleotides and siRNAs (**Table 2**).When the immobilization of the RNA in the nanoformulation is achieved via electrostatic interactions, the release is usually triggered by changes in the electrostatic balance after photocleavage or photoisomerization of a light-sensitive moiety in the nanomaterial. Caging strategies have also been developed for light activation of siRNAs. In such systems, siRNAs are modified with protecting groups, usually cleavable with UV light, that can block the 5’ phosphorylation or recognition by RISC. Meyer and Mokhir have reported uncaging of siRNAs with longer wavelengths by using a protective group sensitive to singlet oxygen and a photosensitizer that generates singlet oxygen once it is irradiated [[48](#_ENREF_48)].

Nanoformulations responsive to UV and NIR light have been described. In general, each nanocarrier contained between 2300 and 8100 RNA molecules [[26](#_ENREF_26),[41](#_ENREF_41),[49](#_ENREF_49)]. Few studies provide information regarding the quantification of cellular nanocarrier uptake. Up to two hundred nanocarriers per cell have been reported [[41](#_ENREF_41)], which released approximately 60 nM of antisense ssDNA after irradiation. While some cells are able to internalize nanocarriers, others may be resistant. For example, results obtained by flow cytometry analyses indicate that a small percentage of the cell population is able to internalize a light-triggerable nanocarrier formulation [[49](#_ENREF_49)]. The release kinetics is dependent in the chemistry of the light-triggerable formulations. It ranged from a few seconds (15 sec, ∼120 fs pulse length) [[49](#_ENREF_49),[50](#_ENREF_50)] up to several minutes (between 2 and 15 min) [[39](#_ENREF_39),[41](#_ENREF_41)] or hours [[26](#_ENREF_26)], with power NIR power intensities ranging from 80 mW [[39](#_ENREF_39)] up to 2.5 W/cm2 [[41](#_ENREF_41),[49](#_ENREF_49),[50](#_ENREF_50)]. The intracellular release of RNA molecules was in general monitored by the knockdown of a targeting gene [[26](#_ENREF_26),[49](#_ENREF_49),[50](#_ENREF_50)].

**4. Advantages and limitations in the use of light-triggerable nanocarriers for the intracellular delivery of biomolecules**

**4.1- Challenges in the intracellular biomolecule delivery**

Many biomolecules are negatively charged at neutral pH and they tend to be cell-membrane impermeable, which is not adequate if intracellular targeting is needed [[51](#_ENREF_51),[52](#_ENREF_52)]. Moreover, endosomal sequestration can also hamper the therapy if cytosolic delivery is required. These issues have elicited the development of more efficient strategies for the delivery of biomolecules [[52-54](#_ENREF_52)]. In case of proteins, the utilization of adjuvants to increase protein uptake and endosomal escape has been reported [53]. Induction of protein internalization by cells with a hypertonic media and release of the protein from the endolysosomal compartment by the use of non-detergent sulfo-betaine has been demonstrated recently. Likewise, the use of endosomolytic agents (e.g. dimerized TAT) has been also reported to facilitate the release of proteins from the endolysosomal compartment into the cytosol [[54](#_ENREF_54)]. However, both approaches (sulfo-betaine and dimerized TAT) have been demonstrated *in vitro* but not *in vivo*. The development of nanocarriers for protein delivery has been also explored (reviewed in refs. [[51](#_ENREF_51)]); however, with limited efficacy (see below). In case of RNA-based therapeutics, several delivery platforms have been developed including the development of nanoformulations to enhance the encapsulation of RNA, intracellular uptake and endolysosomal escape [[52](#_ENREF_52),[55](#_ENREF_55),[56](#_ENREF_56)], the chemical modification of nucleic acids to enhance stability and conjugation with small molecules or peptides for targeting and uptake, namely triantennary N-acetylgalactosamine (GalNac) targeting the liver [[57](#_ENREF_57)] and folate, capable of targeting cancer cells [[58](#_ENREF_58)]. Importantly, so far, a significant part of RNA therapies evaluated in clinical trials was based in the administration of naked RNA or lipid nanocarriers carrying RNAs [[52](#_ENREF_52)].

Although a variety of nanocarriers for protein and RNA delivery has been described there are still many challenges that should be addressed. One of them is related to the targeting of specific tissues and cells. Although some tissues are easily targeted such as the liver, it is hard to have the same success with other tissues. Another challenge is the endolysosomal escape of the formulation. Although multiple formulations have been tested so far, the escape of RNA molecules is still very small (<2%) [[59](#_ENREF_59),[60](#_ENREF_60)]. The use of light-triggerable nanocarriers may overcome some of the obstacles related to targeting and endolysosomal escape. A targeted delivery, i.e., the release of the drug in a specific location may be controlled by the positioning of the laser in the organ/tissue of interest. In addition, enhanced endolysosomal escape of the formulation and/or the biomolecules might be achieved by a rigorous control in the time of the intracellular trafficking (see below).

**4.2- Control of time and space in the biomolecule delivery**

Light-triggerable nanocarriers have been used to control the time in the release of biomolecules within cells. Typically, the activation of the nanoformulation occurs in a few hours (<4 h) after internalization; however, recent studies have demonstrated the feasibility to release proteins [[61](#_ENREF_61)] as well as small molecules [[14](#_ENREF_14)] from one day to several days after nanocarrier internalization.

The spatial control in biomolecule release has been demonstrated mostly *in vitro*, using cells. In this case, transfected cells with light-triggerable nanocarriers have been selectively irradiated using a multiphoton microscope [[32](#_ENREF_32)] or a NIR laser limited by a pattern [[33](#_ENREF_33)]. In some of these studies, single cell resolution has been documented. The *in vivo* spatial control in biomolecule delivery has been demonstrated in the skin [[33](#_ENREF_33)] as well as in tumors [[62](#_ENREF_62)] of rodent models, in the last case after intravenous injection of the light-triggerable nanocarriers.

**4.3- Light-induced endolysosomal escape**

Endosomal escape is many times a significant limitation in intracellular delivery. Consequently, the bioactivity of biomolecules internalized by cells may be hampered by their accumulation in the endosome and consequent hydrolytic and/or enzymatic degradation. Even if the cargo is not degraded, the accumulation in the endosome prevents its biological activity. In order to overcome this problem and ensure cytosolic delivery of the drug, several approaches have been adopted (**Figure 2**). The light activation of formulations may facilitate the endolysosomal escape. Some studies have shown that light irradiation of gold nanocarriers within the endolysomal compartment leads to its escape into the cytosol without impact in cell viability [[41](#_ENREF_41),[63](#_ENREF_63)]. The activation of light-triggerable nanocarriers may contribute for an enhanced endolysosomal escape by damaging the endosomal membrane. For example, the irradiation of photosensitizers immobilized in the nanocarrier may produce singlet oxygen, which in turn induces lipid peroxidation and disrupts the endosomal membrane [[64-66](#_ENREF_64)]. Moreover, irradiation of endocytosed light-triggerable nanocarriers may lead to the rupture of the endosomal membrane by the generation of reactive oxygen species (ROS) [[63](#_ENREF_63)] or by a thermal effect and cavitation [[31](#_ENREF_31)].

**4.4- Limitations regarding the side effects of the light and the accumulation of nanoformulations within cells**

One of the challenges of light- triggerable nanocarriers is related to the potential deleterious effects of light stimuli, which may arise from the direct interaction between light and the tissues or indirectly via the photoactivation mechanism of nanoformulations. These effects depend on several factors, such as the wavelength, power, type of laser source (continuous wave or pulsed laser) and exposure time. Depending on the wavelength, different interactions between light and tissues can occur. UV and visible light are highly absorbed by organic molecules, namely porphyrins that generate reactive oxygen species leading to protein denaturation, DNA damage and lipid peroxidation [[67](#_ENREF_67),[68](#_ENREF_68)]. The effects of NIR light are associated to local heating, which is caused by absorption of light by water molecules, mainly for wavelengths above 800 nm [[69](#_ENREF_69)].

Photoactivation of nanoformulations may also induce toxicity. For example plasmonic nanocarriers, produce a strong photothermal effect when irradiated with light wavelength in the plasmon resonance band [[70](#_ENREF_70)] and they may induce the production of ROS [[71](#_ENREF_71)]. When these formulations are applied in skin and irradiated with a continuous wave (CW) laser they induce the expression of heat shock proteins and promote the migration of inflammatory cells to the implant local [[72](#_ENREF_72)]. However, if the formulations are activated with a pulsed laser there is no significant increase in the temperature of the carrier [[73](#_ENREF_73)] and no effect in the skin [[72](#_ENREF_72)]. The use of upconversion nanocarriers may raise also some issues. For example, the biological effect of UV light generated by these nanocarriers within cells is not well studied. Additionally, in the case of inorganic or polymeric nanocarriers modified with photolabile groups, one must guarantee that the photodegradation byproducts are not cytotoxic. Despite these limitations, the toxicity of light irradiation and the photoactivation mechanisms may be an opportunity for certain cancer applications [[62](#_ENREF_62)].

The interaction between nanocarriers and cells or tissues is also an important aspect that can limit the translation of these systems into the clinic. Specifically, the reports on light-triggered protein or siRNA delivery have been mainly restricted to *in vitro* testing, with very few studies validating light-controlled delivery of these molecules *in vivo* [[33](#_ENREF_33),[62](#_ENREF_62)]. Although several inorganic materials have enabled the use of NIR light for higher penetration depth and less phototoxicity, they have limitations associated to their non-biodegradability. Yet, in some cases, the accumulation of the formulations in the body did not translate into toxicity [[74](#_ENREF_74)].

**5. Future perspectives**

Several light-triggerable formulations have been already approved by FDA for photodynamic therapy involving small molecules such as porphyrin-based molecules as photosensitizers [[75](#_ENREF_75)]. This includes *Visudyne* for the treatment of age-related macular degeneration, *Photofrin* for the treatment of esophageal cancer and endobronchial non-small cell lung cancers and *Levulan Kerastick* for the treatment of actinic kerastoses. In case of proteins and RNA-based therapeutics, more studies are necessary for the clinical translation of light-triggerable formulations. Further efforts should be done in order to develop formulations that respond to light and are biodegradable. In addition, further *in vivo* studies should be performed to evaluate the fully potential of light-triggerable nanocarriers. For small drugs, studies have already shown the *in vivo* potential of light activation [[76](#_ENREF_76),[77](#_ENREF_77)]. We anticipate that light-triggerable formulations for the delivery of proteins and RNA-based therapeutics might have high potential for skin, eye, dental and bowel applications. These tissues offer a good light penetration and there is already some history in the use of light-triggerable formulations for the release of small molecules. For example, light-responsive nanocarriers have been used successfully for the *in vivo* treatment of aged-related macular degeneration after eye injection. The results demonstrated a control in the release time of a small molecule up to 30 days after eye injection, reducing the number of injections needed for the therapy [[78](#_ENREF_78)]. The same advantages may be applicable to biomolecules. Besides skin, eye, dental and bowel applications, other tissues may be reached by the use of fiberoptic devices, which are already in use for the delivery of light in photodynamic therapy [[79](#_ENREF_79)]. Moreover, light-responsive nanocarriers may be an effective strategy to release multiple biomolecules in an orchestrated way which may be important to achieve or enhance a specific biological effect [[61](#_ENREF_61)].

Future studies should further explore the advantages of light-triggerable formulations regarding spatio-temporal control. We anticipate that these formulations could be very useful to investigate the endosomal escape of the proteins/RNA molecules during the intracellular trafficking. The release profile of the nanocarrier may be tuned remotely to facilitate the endolysomal escape. In addition, these formulations may be combined with small- molecule endosomolytic agents to further disrupt the endosomes. Overall, light-triggerable formulations have a great biomedical potential in the delivery of biomolecules and further efforts should be done to explore this potential.

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**REFERENCES**

**1** (2017) The commercial tipping point. *Nat Biotechnol* 35 (3), 181

**2** Leader, B. et al. (2008) Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov* 7 (1), 21-39

**3** Rosenbaugh, E.G. et al. (2010) The Attenuation of Central Angiotensin II-dependent Pressor Response and Intra-neuronal Signaling by Intracarotid Injection of Nanoformulated Copper/Zinc Superoxide Dismutase. *Biomaterials* 31 (19), 5218-5226

**4** Wang, M. et al. (2014) Combinatorially Designed Lipid-like Nanoparticles for Intracellular Delivery of Cytotoxic Protein for Cancer Therapy. *Angewandte Chemie* 126 (11), 2937-2942

**5** Pereira, C.F. et al. (2013) Induction of a hemogenic program in mouse fibroblasts. *Cell Stem Cell* 13 (2), 205-218

**6** Naing, A. et al. (2017) EphA2 gene targeting using neutral liposomal small interfering RNA (EPHARNA) delivery: A phase I clinical trial. *Journal of Clinical Oncology* 35 (15\_suppl), TPS2604-TPS2604

**7** Zimmermann, T.S. et al. (2017) Clinical Proof of Concept for a Novel Hepatocyte-Targeting GalNAc-siRNA Conjugate. *Molecular Therapy* 25 (1), 71-78

**8** Bobo, D. et al. (2016) Nanoparticle-Based Medicines: A Review of FDA-Approved Materials and Clinical Trials to Date. *Pharmaceutical Research* 33 (10), 2373-2387

**9** Mura, S. et al. (2013) Stimuli-responsive nanocarriers for drug delivery. *Nat Mater* 12 (11), 991-1003

**10** Wang, Y.F. and Kohane, D.S. (2017) External triggering and triggered targeting strategies for drug delivery. *Nature Reviews Materials* 2 (6)

**11** Brudno, Y. and Mooney, D.J. (2015) On-demand drug delivery from local depots. *Journal of Controlled Release* 219, 8-17

**12** Rwei, A.Y. et al. (2015) Photoresponsive nanoparticles for drug delivery. *Nano Today* 10 (4), 451-467

**13** Lucky, S.S. et al. (2015) Nanoparticles in photodynamic therapy. *Chem Rev* 115 (4), 1990-2042

**14** Boto, C. et al. (2017) Prolonged intracellular accumulation of light-inducible nanoparticles in leukemia cells allows their remote activation. *Nat Commun* 8, 15204

**15** Henderson, T.A. and Morries, L.D. (2015) Near-infrared photonic energy penetration: can infrared phototherapy effectively reach the human brain? *Neuropsychiatric Disease and Treatment* 11, 2191-2208

**16** Goodwin, A.P. et al. (2005) Synthetic Micelle Sensitive to IR Light via a Two-Photon Process. *Journal of the American Chemical Society* 127 (28), 9952-9953

**17** Gug, S. et al. (2008) Photolabile Glutamate Protecting Group with High One- and Two-Photon Uncaging Efficiencies. *ChemBioChem* 9 (8), 1303-1307

**18** Babin, J. et al. (2009) A New Two-Photon-Sensitive Block Copolymer Nanocarrier. *Angewandte Chemie International Edition* 48 (18), 3329-3332

**19** Barhoumi, A. et al. (2015) Ultraviolet light-mediated drug delivery: Principles, applications, and challenges. *J Control Release* 219, 31-42

**20** Liu, Q. et al. (2017) Phototriggered Drug Delivery Using Inorganic Nanomaterials. *Bioconjugate Chemistry* 28 (1), 98-104

**21** Linsley, C.S. and Wu, B.M. (2017) Recent advances in light-responsive on-demand drug- delivery systems. *Therapeutic Delivery* 8 (2), 89-107

**22** Schmidt, R. et al. (2007) Mechanism of Photocleavage of (Coumarin-4-yl)methyl Esters. *The Journal of Physical Chemistry A* 111 (26), 5768-5774

**23** Bandara, H.M.D. and Burdette, S.C. (2012) Photoisomerization in different classes of azobenzene. *Chemical Society Reviews* 41 (5), 1809-1825

**24** Chen, C. et al. (2011) Photo-responsive, biocompatible polymeric micelles self-assembled from hyperbranched polyphosphate-based polymers. *Polymer Chemistry* 2 (6), 1389-1397

**25** Viger, M.L. et al. (2013) Low Power Upconverted Near-IR Light for Efficient Polymeric Nanoparticle Degradation and Cargo Release. *Advanced Materials* 25 (27), 3733-3738

**26** Yang, Y. et al. (2013) NIR light controlled photorelease of siRNA and its targeted intracellular delivery based on upconversion nanoparticles. *Nanoscale* 5 (1), 231-238

**27** Maity, S. et al. (2014) Spatial temperature mapping within polymer nanocomposites undergoing ultrafast photothermal heating via gold nanorods. *Nanoscale* 6 (24), 15236-15247

**28** Link, S. et al. (1999) Laser Photothermal Melting and Fragmentation of Gold Nanorods:  Energy and Laser Pulse-Width Dependence. *The Journal of Physical Chemistry A* 103 (9), 1165-1170

**29** Fairbanks, B.D. et al. (2011) Photodegradable, Photoadaptable Hydrogels via Radical-Mediated Disulfide Fragmentation Reaction. *Macromolecules* 44 (8), 2444-2450

**30** Saravanakumar, G. et al. (2015) Visible light-induced singlet oxygen-mediated intracellular disassembly of polymeric micelles co-loaded with a photosensitizer and an anticancer drug for enhanced photodynamic therapy. *Chemical Communications* 51 (49), 9995-9998

**31** Braun, G.B. et al. (2009) Laser-Activated Gene Silencing via Gold Nanoshell-siRNA Conjugates. *ACS Nano* 3 (7), 2007-2015

**32** Morales, D.P. et al. (2015) Targeted Intracellular Delivery of Proteins with Spatial and Temporal Control. *Molecular Pharmaceutics* 12 (2), 600-609

**33** Jayakumar, M.K.G. et al. (2012) Remote activation of biomolecules in deep tissues using near-infrared-to-UV upconversion nanotransducers. *Proceedings of the National Academy of Sciences* 109 (22), 8483-8488

**34** Li, J. et al. (2017) Photocontrolled SiRNA Delivery and Biomarker-Triggered Luminogens of Aggregation-Induced Emission by Up-Conversion NaYF4:Yb3+Tm3+@SiO2 Nanoparticles for Inducing and Monitoring Stem-Cell Differentiation. *ACS Applied Materials & Interfaces*

**35** Azagarsamy, M.A. et al. (2012) Photocontrolled Nanoparticles for On-Demand Release of Proteins. *Biomacromolecules* 13 (8), 2219-2224

**36** Jin, Q. et al. (2014) Light-Responsive Polyion Complex Micelles with Switchable Surface Charge for Efficient Protein Delivery. *ACS Macro Letters* 3 (7), 679-683

**37** Foster, A.A. et al. (2015) Light-Mediated Activation of siRNA Release in Diblock Copolymer Assemblies for Controlled Gene Silencing. *Advanced Healthcare Materials* 4 (5), 760-770

**38** Schroeder, A. et al. (2012) Remotely activated protein-producing nanoparticles. *Nano Lett* 12 (6), 2685-2689

**39** Lee, S.E. et al. (2012) Photonic Gene Circuits by Optically Addressable siRNA-Au Nanoantennas. *ACS Nano* 6 (9), 7770-7780

**40** Tortiglione, C. et al. (2009) Fluorescent nanocrystals reveal regulated portals of entry into and between the cells of Hydra. *PLoS One* 4 (11), e7698

**41** Huschka, R. et al. (2012) Gene Silencing by Gold Nanoshell-Mediated Delivery and Laser-Triggered Release of Antisense Oligonucleotide and siRNA. *ACS Nano* 6 (9), 7681-7691

**42** Cho, E.C. et al. (2009) Understanding the role of surface charges in cellular adsorption versus internalization by selectively removing gold nanoparticles on the cell surface with a I2/KI etchant. *Nano Lett* 9 (3), 1080-1084

**43** Shen, J. et al. (2014) Multifunctional gold nanorods for siRNA gene silencing and photothermal therapy. *Adv Healthc Mater* 3 (10), 1629-1637

**44** Wang, B.K. et al. (2016) Gold-nanorods-siRNA nanoplex for improved photothermal therapy by gene silencing. *Biomaterials* 78, 27-39

**45** Carregal-Romero, S. et al. (2012) NIR-light triggered delivery of macromolecules into the cytosol. *Journal of Controlled Release* 159 (1), 120-127

**46** Zhou, L. et al. (2014) DNA-mediated Construction of Hollow Upconversion Nanoparticles for Protein Harvesting and Near-Infrared Light Triggered Release. *Advanced Materials* 26 (15), 2424-2430

**47** Morales, D.P. et al. (2017) Affinity-Based Assembly of Peptides on Plasmonic Nanoparticles Delivered Intracellularly with Light Activated Control. *Bioconjug Chem* 28 (7), 1816-1820

**48** Meyer, A. and Mokhir, A. (2014) RNA Interference Controlled by Light of Variable Wavelength. *Angewandte Chemie International Edition* 53 (47), 12840-12843

**49** Huang, X. et al. (2014) Modular Plasmonic Nanocarriers for Efficient and Targeted Delivery of Cancer-Therapeutic siRNA. *Nano Letters* 14 (4), 2046-2051

**50** Huang, X. et al. (2015) Light-activated RNA interference in human embryonic stem cells. *Biomaterials* 63, 70-79

**51** Gu, Z. et al. (2011) Tailoring nanocarriers for intracellular protein delivery. *Chemical Society Reviews* 40 (7), 3638-3655

**52** Kanasty, R. et al. (2013) Delivery materials for siRNA therapeutics. *Nat Mater* 12 (11), 967-977

**53** D’Astolfo, Diego S. et al. (2015) Efficient Intracellular Delivery of Native Proteins. *Cell* 161 (3), 674-690

**54** Erazo-Oliveras, A. et al. (2014) Protein delivery into live cells by incubation with an endosomolytic agent. *Nat Methods* 11 (8), 861-867

**55** Tan, S.J. et al. (2011) Engineering Nanocarriers for siRNA Delivery. *Small* 7 (7), 841-856

**56** Love, K.T. et al. (2010) Lipid-like materials for low-dose, in vivo gene silencing. *Proceedings of the National Academy of Sciences* 107 (5), 1864-1869

**57** Rajeev, K.G. et al. (2015) Hepatocyte-Specific Delivery of siRNAs Conjugated to Novel Non-nucleosidic Trivalent N-Acetylgalactosamine Elicits Robust Gene Silencing in Vivo. *ChemBioChem* 16 (6), 903-908

**58** Guo, S. et al. (2006) Construction of folate-conjugated pRNA of bacteriophage phi29 DNA packaging motor for delivery of chimeric siRNA to nasopharyngeal carcinoma cells. *Gene therapy* 13 (10), 814-820

**59** Wittrup, A. et al. (2015) Visualizing lipid-formulated siRNA release from endosomes and target gene knockdown. *Nat Biotechnol* 33 (8), 870-876

**60** Gilleron, J. et al. (2013) Image-based analysis of lipid nanoparticle-mediated siRNA delivery, intracellular trafficking and endosomal escape. *Nat Biotechnol* 31 (7), 638-646

**61** Lino, M.M. et al. (2017) Intracellular delivery of more than one protein with spatio-temporal control. *Nanoscale* (in press)

**62** Wang, Z. et al. (2017) Laser-Triggered Small Interfering RNA Releasing Gold Nanoshells against Heat Shock Protein for Sensitized Photothermal Therapy. *Advanced Science* 4 (2), 1600327-n/a

**63** Krpetic, Z. et al. (2010) Inflicting controlled nonthermal damage to subcellular structures by laser-activated gold nanoparticles. *Nano Lett* 10 (11), 4549-4554

**64** Jayakumar, M.K. et al. (2014) Near-infrared-light-based nano-platform boosts endosomal escape and controls gene knockdown in vivo. *ACS Nano* 8 (5), 4848-4858

**65** Cai, C. et al. (2015) Preconditioning Human Cardiac Stem Cells with an HO-1 Inducer Exerts Beneficial Effects After Cell Transplantation in the Infarcted Murine Heart. *STEM CELLS* 33 (12), 3596-3607

**66** Raemdonck, K. et al. (2010) Prolonged gene silencing by combining siRNA nanogels and photochemical internalization. *J Control Release* 145 (3), 281-288

**67** Ramakrishnan, P. et al. (2016) Cytotoxic responses to 405 nm light exposure in mammalian and bacterial cells: Involvement of reactive oxygen species. *Toxicology in Vitro* 33, 54-62

**68** Glickman, R.D. (2002) Phototoxicity to the Retina: Mechanisms of Damage. *International Journal of Toxicology* 21 (6), 473-490

**69** Bixler, J.N. et al. (2014) Assessment of tissue heating under tunable near-infrared radiation. *Journal of Biomedical Optics* 19 (7), 070501-070501

**70** Melamed, J.R. et al. (2015) Elucidating the Fundamental Mechanisms of Cell Death Triggered by Photothermal Therapy. *ACS Nano* 9 (1), 6-11

**71** Pasparakis, G. (2013) Light-Induced Generation of Singlet Oxygen by Naked Gold Nanoparticles and its Implications to Cancer Cell Phototherapy. *Small* 9 (24), 4130-4134

**72** Tang, H. et al. (2013) CW/pulsed NIR irradiation of gold nanorods: Effect on transdermal protein delivery mediated by photothermal ablation. *Journal of Controlled Release* 171 (2), 178-183

**73** Goodman, A.M. et al. (2017) Understanding Resonant Light-Triggered DNA Release from Plasmonic Nanoparticles. *ACS Nano* 11 (1), 171-179

**74** Cheng, L. et al. (2011) In vivo pharmacokinetics, long-term biodistribution and toxicology study of functionalized upconversion nanoparticles in mice. *Nanomedicine* 6 (8), 1327-1340

**75** Christie, J.G. and Kompella, U.B. (2008) Ophthalmic light sensitive nanocarrier systems. *Drug Discov Today* 13 (3-4), 124-134

**76** Li, J. et al. (2016) Near-infrared light-triggered release of small molecules for controlled differentiation and long-term tracking of stem cells in vivo using upconversion nanoparticles. *Biomaterials* 110, 1-10

**77** Cao, J. et al. (2013) Near-infrared light-triggered micelles for fast controlled drug release in deep tissue. *Biomaterials* 34 (26), 6272-6283

**78** Huu, V.A.N. et al. (2015) Light-responsive nanoparticle depot to control release of a small molecule angiogenesis inhibitor in the posterior segment of the eye. *Journal of Controlled Release* 200 (Supplement C), 71-77

**79** Yelin, D. et al. (2006) Three-dimensional miniature endoscopy. *Nature* 443 (7113), 765

**80** Luo, L. et al. (2011) An efficient visible light controlled protein delivery system. *Chemical Communications* 47 (40), 11243-11245

**81** Li, H.-J. et al. (2014) Shell-detachable nanoparticles based on a light-responsive amphiphile for enhanced siRNA delivery. *RSC Advances* 4 (4), 1961-1964

**Table 1 - Examples of light-responsive formulations for protein delivery.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Carrier** | **Cargo** | **Immob. strategy** | **Photo-responsive mechanism** | **Light source** | **Stim.** | **Release Control\*** | **Ref.** |
| Polymeric nanogel modified with metoxy-nitrobenzyl-ether | Alkaline phosphatase and BSA | Encapsulation | Photocleavage-photodegradation of nanogel | CW - UV 365 nm | 10 mW/cm2  2 min | Time (release time: 10 h)\*\* | [[35](#_ENREF_35)] |
| Micelles formed by block copolymer containig *o*-nitrobenzyl groups | FITC-BSA | Electrostatic interactions | Photocleavage | CW – UV 365 nm | 8 W  20 min | Time (release time: 25 h)\*\* | [[36](#_ENREF_36)] |
| Hollow upconversion NPs modiifed with spyropiran | β-Galactosidase | Encapsulation/ electrostatic interaction | Upconversion-isomerization | CW - NIR 980 nm | 0.5W/cm2  20 min | Time and location (release time: 3-12 h)\*\* | [[46](#_ENREF_46)] |
| Polyelectrolyte multilayer microcapsules with AuNPs in the capsule walls | GFP | Encapsulation | Potothermal | NIR 830 nm | 3.8 mW/µm2  < 2 s | Time, location, release of multiple biomolecules (release time: 3- 26 h)\*\*\* | [[45](#_ENREF_45)] |
| Gold nanoshells | GFP | Thiol-gold bond | Photothermal | Pulsed – NIR 800 nm | 8x103­ W/cm2 300 µs | Time and location (release time: min. range)\*\*\* | [[32](#_ENREF_32)] |
| Titania nanoparticles | Hemoglobin | Ti-O coordination bond | Photoinduced electron transformation | Visible light λ>420 nm (Xenon lamp) | 300 W | Dosage (by controlling the irradiation time)\*\* | [[80](#_ENREF_80)] |

Immob.- Immobilization; Stim.- Stimulus; NPs – nanoparticles; AuNPs – gold nanoparticles; CW- continuous wave; UV – ultraviolet; NIR- near infrared; GFP – green fluorescent protein; BSA – bovine serum albumin.\*Type of control demonstrated by the authors for each formulation using *in vitro* or *in vivo* studies. \*\* Studies performed outside the cells. \*\*\* Studies performed within cells.

**Table 2 - Examples of light-responsive formulations for siRNA delivery.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Nanocarrier** | **Immobilization strategy** | **Photo-responsive mechanism** | **Light source** | **Stim.** | **Release Control\*** | **Ref.** |
| Diblock copolymer micelles | Electrostatic and hydrophobic interactions | Photocleavage micelle disassembly | CW- UV 365 nm | 200 mW/cm2  20 min | Time (release time: minutes range)\*\*\* | [[37](#_ENREF_37)] |
| Upconversion NPs | Electrostatic interaction between siRNA and photocaged linker | Light upconversion and photocleavage of the linker | CW – NIR 980 nm | 5.6 W/cm2  2h | Time (release time: 2 hours)\*\*\* | [[26](#_ENREF_26)] |
| Upconversion NPs coated with mesoporous silica | Photocaged siRNA loaded on mesoporous silica surface | Light upconversion and uncaging of siRNA | CW – NIR 980 nm | 2.8 W/cm2  12 min | Time, location, dosage and duration; *in vitro* and *in vivo* studies | [[33](#_ENREF_33)] |
| AuNR | Electrostactic interaction between siRNA and oligofectamine coating on AuNRs | Photothermal effect | CW- 660 and 785 nm | 1.6 W/cm2  15 min | Time and location; *in vitro* studies | [[39](#_ENREF_39)] |
| AuNS | Electrostatic interaction | Photothermal effect | CW - NIR 800 nm | 2.5 W/cm2  2 min | Time and dosage (release time: min. range)\*\*\* | [[41](#_ENREF_41)] |
| AuNS | Thiol-gold bond | Photothermal effect  Thiol-gold bond cleavage | Pulsed – NIR 800 nm | 2.4 W/cm2  10 sec | Time and location (release time: seconds range)\*\*\* | [[49](#_ENREF_49),[50](#_ENREF_50)] |
| AuNS | Thiol-gold bond | Photothermal effect  Thiol-gold bond cleavage | CW- 765 nm | 4 W/cm2  1.5 min | Time, dosage and location; *in vitro* and *in vivo* studies | [[62](#_ENREF_62)] |
| Polymeric nanoparticles | Electrostatic interactions | Photocleavage | CW - UV 365 nm | 10 mW/cm2  5 min | Time (release time: minutes range)\*\*\* | [[81](#_ENREF_81)] |
| Upconversion NPs | Encapsulation in mesoporous silica layer | Photocleavage and uncaging of siRNA | CW- NIR 980 nm | 2 W/cm2  20 min | Time (release time: minutes range)\*\*\* | [[34](#_ENREF_34)] |

Stim.- Stimulus; AuNR – gold nanorod; AuNS - gold nanoshell; CW- continuous wave; UV – ultraviolet; NIR- near infrared. \*Type of control demonstrated by the authors for each formulation using *in vitro* or *in vivo* studies. \*\* Studies performed outside the cells. \*\*\* Studies performed within cells.

**Captions**

**Figure 1** – **Immobilization strategies of proteins and RNA-based therapies in light-responsive nanoformulations**. Immobilization in organic nanocarriers is usually achieved via encapsulation or via hydrophobic or electrostatic interactions between the biomolecule and charged polymers. In inorganic nanoparticles the biomolecules are immobilized on the surface. Upconversion nanoparticles have been reported for the immobilization of negatively charged siRNA using positively charged photocleavable linkers or for immobilization of caged siRNAs. Upconverted UV light is used for the cleavage of the linker and caging compounds. Immobilization on plasmonic nanoparticles has been achieved through electrostatic interactions or directly via thiol-gold bond.

**Figure 2 – Nanoparticle uptake and endosomal escape strategies.** Endocytosed nanoparticles are kept inside endosomes. Then sorting of the cargo occurs, leading to different itineraries. Part of the endocytosed cargo may be recycled back to the membrane and exocytosed. Other part can go through trafficking from early endosomes to late endosomes and lysosomes for degradation. Strategies adopted for endosomal escape include: (1) the use of molecules on the nanoparticle surface for disruption or fusion with the endosomal membrane (this includes polycations, antimicrobial peptides and cell penetrating peptides); (2) photothermal effect (i.e., generation of heat after irradiation of nanoparticles, such as plasmonic NPs, with NIR light) and (3) generation of reactive oxygen species, which lead to oxidation of lipids and the generation of lipid radicals that propagate to other lipids, destabilizing the endosomal membrane.