

Progress Report

Advancement and challenges in multi-domain multi-cargo delivery vehicles

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Reparative and regenerative processes are well-orchestrated temporal and spatial events that are governed by multiple cells, molecules, signaling pathways and interactions thereof. Yet again, currently available implantable devices fail largely to recapitulate nature's complexity and sophistication in this regard. Herein, we discuss success stories and challenges in the field of layer-by-layer, composite, self-assembly and core-shell technologies for the development of multi-domain / multi-cargo delivery vehicles.

1. Introduction

Complex pathophysiologies and regenerative processes involve diverse signaling mechanisms,¹ imposing the need for the development of combinatory therapies comprised of growth factors (GFs),² drugs,³ genetic material⁴ and cells.⁵ Multi-compartment and multi-cargo sustained and localized delivery vehicles have already shown promise in microbial infection,⁶ cancer treatment,⁷ wound healing⁸ and regenerative medicine.⁹ Such advanced therapies surpass limitations¹⁰ associated with conventional administration approaches, such as insufficient target selectivity;¹¹ incompatible *in vivo* pharmacokinetics and distribution profiles between different bioactive molecules;¹² unpredictable accumulation ratios and interactions;¹³ short circulation times and half-lives;¹⁴ and even harmful systemic side effects.¹⁵

Early delivery designs aimed primarily at controlling the bioavailability through either cleavable linkers¹⁶ or by promoting diffusion and degradation of the carrier.¹⁷ Current delivery systems aspire to provide controlled, temporal and spatial delivery.¹⁸ Further, they seek to protect the cargos,¹³ offer higher solubilization ratios of the encapsulated molecules¹⁹ and allow sequential or combined release.²⁰ For successful development of such elegant systems, considerable challenges should be addressed, including cargos' solubility, size and molecular weight,²¹ as well as the cargo's type, as the successful co-delivery of hydrophobic and hydrophilic molecules is a far more daunting task than just delivering similarly soluble molecules.²¹ Further, it is of paramount importance to control the architecture of the delivery vehicles over the various scales, as this will determine the quantity and bioactivity of the cargos that can be delivered.²² The functionality of the carrier, through incorporation of stimuli-responsive motifs, targeting moieties and cleavable linkers should also be incorporated into the device design.²³

In this manuscript, we discuss advancements, limitations and future perspectives in layer-by-layer (LbL), self-assembly, composite and core-shell scaffold fabrication technologies for the co-delivery of multiple cargos.

2. Layer-by-layer technologies

LbL scaffold fabrication technology allows the development of multi-layered thin films (**Figure 1**). It was first introduced in 1991 and enabled the formation of a thin film by depositing alternating layers of oppositely charged materials.²⁴ Since then, numerous alternative formation forces (e.g. hydrophobic interactions,²⁵ biological interactions,²⁶ hydrogen bonding²⁷ and covalent bonding²⁸) and fabrication methods,²⁹ have been developed for tissue engineering³⁰ and control drug delivery / release,³¹ using a diverse range of materials for different clinical indications (**Table 1**).

It is worth noting that synthetic and natural polymers are traditionally used in the assembly of multilayer films. The choice of the materials is dependent on the molecular interactions governing the films assembly.^{29b} Electrostatic interaction is restricted to the fabrication of charged and water-soluble multilayer materials, whilst covalent bonding, which is a highly desirable option as it leads to more stable films, is merely available for molecules with complementary functional groups. In any case, synthetic polymers³² are most commonly used for the preparation of multilayered films because of their versatility and tunable properties. Polyelectrolytes have also been utilised for dual delivery of biologics,^{32a} however, cytotoxicity of synthetic polyelectrolytes remains of concern.³³ Although incorporating them as primer layer could decrease their toxicity,³⁴ it can still be triggered afterwards from their degradation products. In particular, synthetic polycations have been reported to induce cell death as a result of interactions with the negatively charged surface of the cell membrane.³⁵ For these reasons, natural polymers³⁶ are under intense investigation and they have shown promising results for several indications, including multi-drug delivery.^{36a}

The technologies used to assemble such multi-layered films are based on the principles of immersive,³⁷ spin-coating,³⁸ spray,³⁹ fluidic⁴⁰ and electromagnetic assembly.^{41, 29a} Immersive assembly involves the immersion of a substrate into solutions of the desired materials and

subsequent washing away the excess. Although this fabrication method allows coating of substrates with complex architectural features, possible residuals from each deposition step and laborious washing steps limit its applicability.⁴² Spin-coating and spray assembly LbL procedures have emerged as promising alternatives to the conventional immersive assembly due to manufacturing low costs and reduced processing time, thereby being more suitable for industrial applications. Spin-coating utilizes rotating substrates to deposit uniform layers and remove excess coating material. The majority of spin-coating assembly is performed by either casting the solution onto a spinning substrate or casting the solution onto a stationary substrate that is then spun.³⁸ This technology is generally limited to coat flat surfaces, but it is faster than immersion assembly and results in more homogenous and multi-layered films.⁴³ Spray assembly allows the development of films with more distinct layers than those derived from immersion assembly techniques.⁴⁴ They are assembled by aerosolizing polymeric solutions and sequentially spraying them onto planar or non-planar substrates, which can be rotating to ensure a more homogeneous coating.³⁹ More recently, fluidic and electromagnetic assemblies have been explored, but their applicability is still limited due to complex and still primitive infrastructure requirements.^{29a} Fluidic assembly allows region-specific patterning, by inserting a geometric chamber over the substrate and then flowing the solution through it. This technique has also enabled LbL assembly of sensitive substrates (e.g. phospholipid membranes) with defined compositional asymmetry and lamellarity.⁴⁵ Electromagnetic assembly utilizes electric or magnetic fields to deposit polymeric layers that are generally thicker than the ones prepared with the aforementioned techniques.⁴⁶

By selecting polymer components and controlling film deposition parameters, LbL films can be rationally designed to act as a platform for controlled delivery of multiple bioactive agents for multiple clinical indications. LbL assembly offers several advantages, including simultaneous delivery of multiple and heterogeneous cargos, such as hydrophobic and hydrophilic drugs,⁴⁷ opposite charged molecules,⁴⁸ nucleic acids and/or cells;⁴⁹ opportunity to

functionalize the outer layers with active targeting moieties^{32c} or stimuli-responsive motifs;⁵⁰ possibility to decrease the cytotoxicity of substrates, such as semiconductor nanoparticles (NPs) by depositing layers of biocompatible polymers;⁵¹ temporal control release of different cargos by modulating the thickness and the number of the layers.^{52, 32c} The cargo can be conveniently loaded into LbL-assembled films by direct assembly^{32a} or post-diffusion.⁵³ The latter, is a convenient option that relieves concerns over drug storage in the films; it is limited though to low molecular weight drugs, which can diffuse easily through the pores of the film.⁵⁴ The cargos can be released by triggering an external stimulus⁵⁵ or after the film components degradation.⁵⁶ Further, the amount of cargo loaded can be controlled by adjusting the numbers of layers applied.⁵⁷

LbL films can be deposited and subsequently released from substrates to produce free-standing films by dissolving a pre-deposited sacrificial layer between the substrate and the film⁵⁸ or by an ion-triggered exfoliation method.⁵⁹ However, the amount of molecules that can be loaded is limited by the ultrathin nature of the films and their nm scale thickness makes them susceptible to mechanical damage (e.g. rupture).⁶⁰ Nonetheless, multi-cargo multi-compartment μm -thick implantable devices have been developed: spin-coating was utilized to develop a bilayer film loaded with a GF on top of a LbL film loaded with an antibiotic via a post-diffusion process.⁵⁴ The versatility of LbL self-assembly technique allows also the formation of 3D systems by using templates, instead of flat surfaces, that have higher surface area and improved cell accessibility when compared to planar substrates.⁶¹ The assembled films can be adsorbed onto a number of different 3D templates, for example degradable polymeric NPs,⁶² liposomes,⁶¹ carbon nanotubes,⁶³ mesoporous silica.⁶⁴ Charged polypeptides or synthetic polyions can be introduced in alternating assembly to create an ionic film outer layer, which can be used to control drug release from the internal core, producing a core-shell design. LbL systems can be considered the simplest vectors for gene delivery.⁶⁵ Multilayer films can be easily fabricated by exploiting the electrostatic forces between DNA or RNA (anionic building blocks) and any

cationic polymer.⁶⁶ The ability to perform LbL coatings at physiological conditions also enabled cell surface coating,⁶⁷ encapsulation⁶⁸ and delivery,⁴⁹ as well as the creation of a suitable niche for stem cell differentiation⁶⁹ by the co-delivery of GFs for specific lineage differentiation.

The main limitation that has been reported in LbL multi-layered structures is the interlayer biomolecule diffusion⁷⁰ that depends on the film's internal structure and nm scale porosity, which might lead to the mixing of the entrapped cargos.⁷¹ This elicits the importance of investigating physical erosion mechanisms and changes in film morphology that could occur during the film degradation or after the films are exposed to physiological media, leading to interlayer diffusion and changes in the internal structures.^{31a} Between innovative approaches that have been explored to limit this issue, graphene oxide⁷² and laponite clay⁴⁸ were effectively used as barrier interlayer to block inter-layer diffusion.⁴⁸ Another interesting approach was based on the use of antagonists in the individual layers (e.g. vascular endothelial GF (VEGF) and anti-VEGF) to create spatially confined compartments of the cargos through diffusion blocking.⁷³

Although LbL technologies have exponentially grown in recent years, no clinical trials have been conducted yet (Source: clinicaltrials.gov; Terms searched: 'layer-by-layer', 'multi-layer'). Current research is moving away from the random diffusion-driven kinetics for layer deposition and heading towards the development of faster kinetics⁴² and more robust and more reproducible fabrication procedures through automation.^{37, 74, 30b} High quality free-standing LbL films have also been fabricated with a modified spin-coating assembly method at substantial reduced manufacturing time, avoiding laborious substrate handling and washing steps.⁷⁵ In the years to come, scalability issues would be addressed in a more comprehensive manner, allowing the manufacturing and clinical translation of more elegant multi-compartment delivery systems for controllable multiple-cargo delivery.

3. Self-assembly technologies

Self-assembly technologies rely on the presence of amphiphilic domains (possessing a polar and a non-polar region) and a natural thermodynamic predisposition to form independent hydrophilic and hydrophobic multilayer compartments.⁷⁶ When exposed to a hydrophilic solvent, the polar part orients itself towards the solvent, whilst the non-polar part orientates itself away from the solvent; the opposite occurs in an hydrophobic solvent.⁷⁷ This separation not only enables the encapsulation of multiple cargos, but also allows for independent release thereof, thus surpassing some of the limitations (mixed cargo release, which can compromise the therapeutic effect of encapsulated cargos; required high drug concentrations and unselective cargo release, which can cause side effects and cytotoxicity in normal tissues) of conventional delivery vehicles.⁷⁸ Liposomes,^{76, 79} polymersomes,⁸⁰ dendrimers⁸¹ and polymeric micelles,^{77, 82, 81e} all formed through self-assembly processes, have been used to create controlled release multiple cargo (e.g. hydrophilic and hydrophobic molecules, GFs, genetic material, imaging contrast agents) compartmentalized delivery vehicles (**Figure 2**) for multiple biomedical applications (**Table 2**).

Liposomes are nano/micro self-assembled spherical colloidal vesicles which consist of a hydrophobic phospholipid bilayer surrounding an aqueous hydrophilic core.⁸³ Although liposomes were first described in 1965⁸⁴ and their potential application as drug delivery vehicles was shown in 1971,⁸⁵ their application in a clinical trial came to fruition in 1989.⁸⁶ To-date, several iterations of liposomes (e.g. solid phase bilayer liposomes,⁸⁷ ‘stealth’ liposomes,⁸⁸ targeted liposomes⁸⁹ or even triggered release liposomes⁹⁰) are available. These systems aim to increase drug loading capability and bioavailability and to offer better controlled release rate of the encapsulated molecules.^{91, 76} They also aspire to prevent recognition by the immune system, including activation of the complement system and macrophage clearance⁹² and to decrease systemic cytotoxicity⁹²⁻⁹³ through their high content in natural phospholipids that tends to minimize possible toxic side effects.⁹⁴

One of the most used techniques to prepare liposomes is the creation of a thin film through sonication, rotary evaporation and mix extrusion, followed by rehydration and drug loading.⁹⁵

⁶⁸ Another commonly used technique for liposome preparation is dual asymmetric centrifugation, which involves two axes of centrifugation, one of them being the sample's own vertical axis, constantly forcing the sample material toward the center of the centrifuge, leading to a more efficient homogenization.⁹⁶ Other methods of preparation include surface functionalization with reactive groups^{78, 97} or with cross-linking of different multi-lamellar compartments.⁹⁸ Drug encapsulation can be accomplished through passive or active loading. Passive loading can be achieved by solubilizing the drug in the lipid solution during liposome formation,⁹⁹ whilst active loading uses chemical gradients to encapsulate drugs.¹⁰⁰ Although active loading is more effective in achieving higher drug-to-lipid encapsulation ratio and allows a more controlled drug release, it could lead to low drug availability at the site of action.¹⁰⁰⁻¹⁰¹ Methods of active loading include the use of ammonium sulfate^{95a} or calcium acetate¹⁰² gradients for weak bases and acids, respectively. Cyclodextrin,¹⁰³ EDTA¹⁰⁴ and transition metals¹⁰⁵ can be used for the formation of poorly soluble or even insoluble precipitates inside the liposome. Water-insoluble molecules can be loaded through a pro-drug formation / derivatization process.¹⁰⁶ Despite the significant advancements in the field of liposomes, only recently multiple cargo / co-delivery approaches using single liposome formulations have been deployed,^{95b, 107} albeit with moderate levels of success. For example, although the produced systems achieved high drug-loading ratios, localized delivery / release of the encapsulated cargos and significant decrease in tumor viability *in vitro*, they did not demonstrate the predicted efficacy *in vivo*.^{107d} Such drawbacks can be attributed to technical and stability issues, batch-to-batch variability, particle size control, sterilization method used and traceability of the lipid composition of each individual formulation.¹⁰⁸ Some types of formulations might also prove to be more problematic to implement, as is the case of cationic formulations, which can be cytotoxic⁹³ and the use of unsaturated fatty acids, which can be easily oxidized.^{108b}

Polymersomes borrow the same rationale from liposomes, as they are also constituted of both hydrophobic and hydrophilic domains, forming amphiphilic vesicles from synthetic block polymers.^{80d} This class of vesicles is comprised of a bi-layer membrane of hydrated hydrophilic coronas that are in contact with an aqueous core and medium, both on the inside and the outside of a hydrophobic core.^{80b} Although many synthetic¹⁰⁹ and natural^{80d} polypeptides can be used on their own to create amphiphilic block copolymers, the most interesting approaches tend to combine them to get the most advantageous features from both (e.g. solubility, processability, elasticity versus secondary structure, functionality and cytocompatibility).^{110, 80d} Obviously, during the selection of the polymers to be used, general considerations regarding biodegradability, water solubility, immunogenicity and interactions must be considered.^{110c, 111} Polymersomes are synthesized through ring-opening polymerization,^{97d} either using a solvent exchange method,¹¹² or, similarly to liposomes, through a thin film formation and rehydration,¹¹³ with cargo conjugation usually being done through dialysis,^{97d} pH gradient,¹¹⁴ or salt gradient.¹¹⁵ Polymersomes are considerably more stable than liposomes,¹¹⁶ capable of longer circulation times,¹¹⁷ more mechanically robust¹¹⁶ and carrying larger quantities of both hydrophobic and hydrophilic molecules,^{80a} thus proving to be a viable alternative to liposomes for multiple cargo delivery.¹¹⁸ Polymersomes also possess lower immunogenicity and cytotoxicity than liposomes,^{111, 119} in part due to the use of biodegradable non-toxic synthetic polymers¹²⁰ or non-immunogenic natural polymers.¹²¹ Surface functionalization can reduce further potential immune response.¹²²

Admittedly, polymersomes are characterized by a slower rate of release of their encapsulated cargos, when compared to liposomes, which can compromise the therapeutic efficacy of the encapsulated bioactive agents.^{115, 123} To surpass this limitation, several groups have tried to create tunable systems that release their cargo in a stimuli-responsive (e.g. pH,¹²⁴ glucose levels,¹²⁵ presence of cysteine,¹²⁶ light,¹²⁷ heat,¹²⁸ glutathione,¹¹⁸ magnetic fields¹²⁹) fashion. Another issue that needs to be addressed is the lack of specific cellular interactions and

targetability by some of the peptide-based polymersomes,^{80d} which can be surpassed through surface functionalization with targeting reactive groups or by using natural polymers with native biological interaction capabilities.^{80d} Several polymersome formulations have been used for the co-delivery of bioactive agents *in vitro*^{130a-c, 118, 130d, 114, 130e} and *in vivo*,¹³¹ achieving satisfactory results regarding bioactive agent encapsulation, localized delivery, sustained release, tumor prevention activity and low levels of systemic toxicity. However, polymersomes have yet to reach the clinic, indicating that further studies regarding their safety and efficacy are needed.

Dendrimers are a class of synthetic macromolecules with a tree-like structure, synthesized in a stepwise manner from branched monomer units.^{81a, 132, 81d} These hyper-branched and monodisperse multivalent nano-systems possess several characteristics that make them ideal for drug delivery,^{81c} including well-defined molecular weight and macromolecular structure,¹³³ uniform size,¹³⁴ available internal cavities with high loading capacity,¹³⁵ favorable pharmacokinetics¹³⁵ and a high density of surface active (amine) groups, ideal for functionalization and targeting.¹³⁶ Among the available polymeric dendrimers, polyamidoamine (PAMAM) dendrimers have been used extensively for drug delivery.^{132f} PAMAM dendrimers are synthesized from an initiator core, followed by sequential branching, with the process being repeated throughout each generation, leading to increasingly branched structures, which can then be used as molecule storing compartments, usually through a two phase dialysis process.¹³⁷ Despite their promising characteristics (e.g. high loading capacity, favorable pharmacokinetics and high concentration of active surface groups, ideal for functionalization), their potential for clinical translation has been hampered due to cytotoxicity issues associated with their positively charged amino groups¹³⁸ or as a function of generation.¹³⁹ To this end, surface functionalization strategies are under intense investigation to increase their biocompatibility and circulation time,^{81c, 132e} to increase targeting capabilities^{132b, 132c} or to add stimuli (e.g. pH¹⁴⁰) responsiveness.

Polymeric micelles are amphiphilic copolymeric nanostructures formed by a thermodynamically driven self-aggregation process above the critical micellar concentration, which is the concentration beyond which a surfactant can form micelles.¹⁴¹ Similarly to other described amphiphilic NPs, they can form two separate hydrophilic and hydrophobic compartments depending on the solvent in which they are mixed.^{82d} In contrast to other polymeric NPs, they are thermodynamically reversible,¹⁴² which can present some disadvantages for drug delivery, such as cargo precipitation after abrupt micellar dilution.¹⁴³ On the other hand, it enables them to be tailored to respond to different micro-environmental conditions and better control the pharmacokinetics and release profiles of the encapsulated cargos by mixing different amphiphilic monomers and cross-linking them.¹⁴⁴ The degree of cytotoxicity, biodegradability, water solubility and immunogenicity is dependent on the choice of the polymeric building blocks, and should be considered during the design phase.¹¹¹ Initial approaches, involving polymeric micelles for the delivery of multiple bioactive cargos, consisted of basic copolymeric micelles encapsulating multiple chemotherapeutic agents.¹⁴⁵ More refined approaches have been recently described, with the development of redox^{140b} and pH¹⁴⁶ sensitive micelles, targeted systems¹⁴⁷ or micelles functionalized with biodegradable linkers.¹⁴⁸ Despite the promising results of micelle system for co-delivery of bioactive agents *in vitro*¹⁴⁹ and *in vivo*¹⁵⁰, these systems have only been used in clinical setting for the delivery of single bioactive agents.^{82a} Self-assembled systems, primarily liposomes and micelles, have been used extensively in clinical trials for single molecule (e.g. bupivacaine,¹⁵¹ sodium hyaluronate¹⁵² and cytarabine¹⁵³ with liposomes; radioactive ligands¹⁵⁴ with dendrimers; doxorubicin,¹⁵⁵ paclitaxel¹⁵⁶ and cisplatin¹⁵⁷ with micelles) delivery (Source: clinicaltrials.gov; Terms Searched: ‘liposome’, ‘polymersome’, ‘dendrimer’, ‘micelle’; Number of Studies: 2,039, 0, 1, 32, respectively) due to their highly tunable properties that allow sustained and localized delivery of their cargos. To-date, none of these systems has been used for multi-cargo delivery in clinical setting, clearly

illustrating the need for further research on their bioactivity and safety.^{82c} Considering that these systems have become relatively easy to scale-up, with emerging production techniques, such as ethanol injection,¹⁵⁸ use of vertical stirred tank reactors¹⁵⁹ and micellar electrospray⁹³ showing narrow size distribution, good reproducibility and appropriate stability, in the years to come, advancements in chemistry and functionalization are expected to address cytotoxicity and adequate cargo loading / protection issues,¹⁵⁸⁻¹⁶⁰ bridging that way the gap between proof of concept and clinical translation.

4. Composites technologies

Composites are materials obtained through the combination of constituents with different macrostructures, which result in the creation of individual compartments, ideal for the delivery of multiple bioactive agents.¹⁶¹ Composites were developed to overcome shortcomings of single constituent materials and to obtain synergistic benefits (e.g. improve loading and release kinetics of bioactive agents) by combining multiple materials.¹⁶² To-date, composite systems^{161, 163, 162} are used extensively in the biomedical field as bioactive agent delivery vehicles (**Table 3**). The most prominent combinations (**Figure 3**) include hydrogel systems loaded with several types of particles¹⁶⁴ or fibers;¹⁶⁵ porous scaffolds with integrated particles;¹⁶⁶ and fibers doped with particles.¹⁶⁷ Considering the vast range of materials that can be combined, the optimal choice will always be application dependent.

Hydrogel systems¹⁶⁸ are frequently loaded with particles,^{164, 169} micelles¹⁷⁰ and fibrous materials.^{165, 162} Carbon,¹⁷¹ polymeric,¹⁷² inorganic/ceramic¹⁷³ or metal/metal-oxide¹⁷⁴ in nature NPs result in superior physicochemical, electrical and biological properties¹⁷⁵ due to the enhanced surface interaction between the NPs and the polymer chains of the hydrogel system. The type of cross-linking (chemical or physical) should also be considered, as it would affect mechanical properties, cargos' release profile and cytotoxicity.¹⁷⁶ Hydrogel systems that encapsulate mixed particle populations can modulate the release kinetic of the different

cargos.^{145, 177} The incorporation of inorganic magnetic NPs has allowed the modulation of external temperature, either for hyperthermia treatments or for a stimuli-dependent (e.g. temperature or magnetic field) cargo release.^{132e} Additionally, the observed volumetric change, after the incorporation of the NPs, leads to repeatable swelling-deswelling cycles and consequently on-off controlled cargo release by an external magnetic field.¹⁷⁸ Through combination of micellar particles and a hydrogel system, it is possible to create a multi-delivery system that conjugates the appropriate hydrophobic loading properties of micellar particles and the hydrophilic loading and tunable release capacities of the hydrogel system.¹⁷⁹ Furthermore, it is possible to functionalize these systems to be responsive to environmental factors (e.g. pH¹⁷⁹⁻¹⁸⁰ or temperature¹⁸¹), thus increasing their specificity and potential as controlled delivery devices.

One of the methods used to obtain composites of hydrogels / electrospun fibers is by physically combining them in a multilayered structure by LbL technologies.¹⁸² The large surface area of the fibers which ensures a high loading, whilst the hydrogel, through its swelling, cross-linking and degradation rate properties, allows controlled release of the cargo.¹⁸³ The fibers, in addition to their mechanical reinforcement¹⁸⁴ and directional cell growth¹⁸⁵ role, slow down the cargo diffusion into the hydrogel, which in turn tunes the release kinetics.¹⁸⁶ Likewise, cross-linked hydrogels can act as barrier to the potential initial burst release from the electrospun fibers. The option of doping particles to electrospun fibers has been largely explored in the last decade for multiple applications.^{187, 167} The loading of multiple cargos into this class of composites allows for an earlier and faster release from the fibers and a slower cargo release from the NPs.¹⁸⁸ This class of composites has been prepared by directly mixing a polymer solution with NPs suspension,¹⁸⁸ by sol-gel/electrospinning¹⁷⁵ or by electrospinning / electrospaying.¹⁸⁹

Porous systems (e.g. sponges) have been used in conjugation with different particulate systems (e.g. microspheres^{190, 166} or microparticle¹⁹¹) allowing the localized co-delivery of multiple cargos. Their extensive porous network guides blood vessel formation promoting

angiogenesis¹⁹² and allows osteoconduction, osteoinduction and osteogenesis,¹⁹³ all key factors in bone repair and regeneration.¹⁶⁶

Whilst numerous single cargo-composite technologies have been clinically translated or even commercialized, multi-cargo composite-based systems have yet to reach the clinic (Source: clinicaltrials.gov; Terms Searched: ‘composites’, ‘composites AND delivery’, ‘composites AND release’, ‘hydrogel AND particle’, etc.). Although multi-component / multi-cargo composite systems provide superior properties to their single counterpart component systems, the complexity involved in optimizing the desired composition, the spatial distribution and the temporal controlled delivery and release of the multiple cargos still need to be addressed. Factors, such as the nature of the materials, interaction of the materials, individual and combined toxicity, *in vivo* biodegradability and manufacturing / scalability should be considered in the device design stage.¹⁹⁴ Considering that the individual elements, in most cases, have already been clinically translated for single cargo delivery (e.g. hydrogel¹⁹⁵ or porous scaffold¹⁹⁶ based delivery), it is expected that in the near future more multi-cargo composite technologies would reach clinical translation and commercialization.

5. Core-shell technologies

Core-shell (**Figure 4**) methods¹⁹⁷ have been used extensively in regenerative medicine¹⁹⁸ for multiple cargo controlled delivery (**Table 4**). Two well-defined structures, a core inner layer surrounded by a shell outer layer, secure and partition multiple bioactive agents into different compartments at relevant quantities, preserving their stability. The newly formed combined structure enables different release rates, since the cargo in the core is released more slowly / later than the one encapsulated in the shell molecule, whose release can be tailored by modifying the shell composition and thickness. The core and the shell can be conceived as dense or porous, according to the final application.^{197b} Dense shells are usually made of metal oxides (e.g. silica and alumina) that exhibit hydrophilic behavior or non-metals (e.g. carbon)

that have hydrophobic character. Dense shells are primarily used in sensors,¹⁹⁹ photo-thermal / chemotherapy strategies²⁰⁰ and batteries.²⁰¹ Porous shells, obtained by the addition of surfactant molecules during the shell formation, can accommodate molecules and enable their diffusion. This allows their use in drug delivery,²⁰² photo-thermal / chemotherapy strategies²⁰³ and imaging.²⁰⁴ To-date, various shapes (e.g. fibers,²⁰⁵ spheres²⁰⁶) and wide range of sizes (from nm²⁰⁷ to μm ²⁰⁸) and materials (metals,²⁰⁹ quantum dots,²¹⁰ polymers²¹¹) have been proposed for multiple cargo delivery.

Core-shelled systems, mainly NPs, can be fabricated via chemical reactions (e.g. chemical vapor deposition) or post-shelling methodology.²¹² An alternative strategy is based on the electrospinning method²¹³ that gives rise to fibrous scaffolds by using electric forces in a random or uniaxial oriented manner, with nano- to micro- scale topography and with controlled porosity.²¹⁴ However, fibers deriving from a conventional electrospinning process do not ensure homogeneous distribution of the cargo and surface-based delivery is often burst in nature.¹⁹⁸ An improvement in this direction has been made with the introduction of the co-axial^{207, 167c, 215} and emulsion^{69, 216} electrospinning. Co-axial electrospinning allows single-step formation of core-shell fibers²¹⁷ or hollow fibers after a second step of heat treatment or chemical withdrawal.²¹⁸ Co-axial electrospinning is a modification of the traditional single spinneret electrospinning set up, that is replaced by two coaxial capillaries. It employs electric forces acting on polymer solutions and results in significant stretching of polymer jets due to direct pulling. The set-up includes an inner and outer nozzle arranged in a concentric geometry able to pump two polymer solutions or a polymer solution (shell) and a non-polymeric liquid or powder (core) simultaneously to initiate the core-shell jet.²¹⁹ This technology allows also the introduction of the cargo directly into a core made from fluids, like water or aqueous polymeric solutions, in order to better preserve their bioactivity.²²⁰ Co-axial and tri-axial electrospinning offer control over release kinetics by altering the fiber thickness or the molecular weight of the core polymer.²²¹ The addition of polyethylene glycol in the shell layer can also offer control

over the cargo's release.²²² Biofunctionalization of the shell, via surface immobilization with biological motifs, can also be performed to provide specific interaction of the scaffold with cells and increase the bioactivity of the system.^{223a, 207, 223b} However, this process usually requires multi-steps of chemical modifications of the surface at wet state, which might disturb the core-shell fiber configuration because of a softer core. Another limitation is that by using hygroscopic materials (e.g. gelatin, collagen, peptides) that are typically preferred due to their excellent cytocompatibility water molecules form channels between the fiber core and the shell. As a consequence, the release from the core occurs rather rapidly.²²⁴ To solve this issue, three-layer (core/ intermediate/shell) fibers have been produced by tri-axial electrospinning, allowing the loading of water-soluble molecules in the outer layer, without causing a premature release from the molecules in the core layer.²²⁵ To date, there are only few proof-of-concept *in vitro* studies²²⁵ using tri-axial electrospinning for multiple release, possibly because of the challenges of this new technology and the number of coupled parameters governing the process and the properties of the resultant fibers. Co-axial or tri-axial fibers can be fabricated with multiple polymers, both natural and synthetic in origin.^{221b, 226} Whilst synthetic materials are often preferred due to their good processability and favorable mechanical properties, the use of natural polymers appears to be appealing, as they mimic the natural composition of the extracellular matrix (ECM). However, it has been reported that when trying to process natural polymers, such as collagen,²²⁷ hyaluronic acid²²⁸ and fibrinogen²²⁹, into an electrospun scaffold, many of their structural and biological properties are lost. Specifically, the use of solvents and the intense electrostatic field represent the major threats for biopolymers. To take advantage of the favorable processing and tenability of the synthetic materials with the biological advantages of the natural polymers, post-processing surface functionalization is frequently employed.²³⁰ Emulsion electrospinning has also attracted great interest to produce core-shell fibers for controlled release of multiple cargos. The emulsion contains an oil phase, obtained by dissolving polymers into organic solvents that can incorporate hydrophobic agents, and a water

phase containing hydrophilic drugs or proteins.^{69, 231, 216} The major advantage of emulsion electrospinning over the co-axial method is the infrastructure simplicity.²³² However, the use of an emulsified agent constitutes a major drawback, as it is difficult to remove it completely and might lead to protein denaturation.²¹² Interestingly, combination of co-axial and emulsion electrospinning have been reported,²³³ where the core was made by co-axial electrospun fibers and the shell was made by co-axially emulsion electrospun fibers; the system allowed controlled and temporal release of multiple cargos by adjusting the composition and density of the core. Among alternative techniques that have been used to fabricate core-shell fibers, it is worth to mention the template deposition method,²³⁴ the phase separation method,²³⁵ the co-concentric extrusion^{197a} and microfluidics.²³⁶ Natural polymers, such as collagen²³⁷ and alginate,²³⁸, have been successfully tested with these methods. An elegant system for the delivery of multiple types of cells has been developed by using a combination of microfluidics and co-centric extrusion.²³⁹ The system was able to encapsulate cells into different proteins, which served as a core layer, using alginate as a shell. However, fiber formation was only successful when the cells were cultured in relatively stiff substrates, which clearly illustrates the importance of the material for sustained / controlled delivery of cells. Considering that template deposition is a two-step method, microfluidics systems are associated with higher in fabrication and operation costs and complexities and emulsion electrospinning utilizes an emulsified agent that can ultimately lead to encapsulated protein denaturation, co-axial electrospinning remains the most extensively used method, as it generates core-shell fibers in a single-step and allows the encapsulation and the protection of even unstable cargos in an aqueous environment.

Core-shell spheres, particles and capsules have also been developed for multiple cargo delivery. Their fabrication has been achieved with numerous chemical and physicochemical methods, including: coaxial electrodropping,²⁴⁰ LbL assembly,²⁴¹ emulsion polymerization,²⁰⁸ microfluidics,²⁴² surface-modification of the core,²⁴³ emulsion polymerization,²⁴⁴ and co-axial electrospraying.²⁴⁵ Core-shell NPs offer the possibility of coating the core with a shell made of

a more biocompatible material.²⁴⁶ The coating can also increase hydrophilic behavior, offer external control (e.g. via magnetic or thermal mechanism) to the process of drug targeting and provide better dispersibility and binding affinity to receptors and ligands for targeted delivery.^{146a} Microparticles and microcapsules with core-shell structure have been mainly used to encapsulate and deliver cells; the core holds the cells and provides them with a 3D culture environment.²⁴⁷ Whilst the composition of the core is crucial as it determines the fate of the encapsulated cells,^{248, 247c} the shell protects the inner biological ingredients and governs their release kinetics as a function of the layer thickness, the loading location and the density of the encapsulated cells.^{213c}

Technologies to develop core-shell fibers and particles from various polymers are developing at a fast pace. Although there are many promising basic research and preclinical studies, the use of core-shell fibers has yet to reach the clinic. However, at clinicaltrials.gov (Term Searched: 'electrospinning') 3 trials were identified, 2 of which have been completed and involved the development of a nitric oxide delivery multilayer transdermal patch for the treatment of cutaneous leishmaniasis²⁴⁹ and diabetic foot ulcers.²⁵⁰ Although none of these trials assessed a multi-delivery device, they constitute a significant progress in the field of drug delivery via electrospun scaffolds. In the years to come, many of the fundamental interactions between the cargos and the carriers would be better understood, better structures with tailored release capacity would be developed and more elegant functionalization systems would be established to enable clinical translation and commercialization of these elegant technologies. For example, a recent study has already demonstrated an one-step route towards surface bio-functionalization using an electric field-driven surface enrichment of the electrospun fibers.²⁵¹ With respect to scalability, the slow production rate²⁵² should also be addressed anon. Towards this goal, multi-needle²⁵³ and needless²⁵⁴ technologies are coming up to industrialize the fabrication rate.²⁵⁵ Within the multi-needle technologies, it is worth noting a method that accomplished high production rate of uniform fibers using an hexagon shaped multi-needle spinneret; the

conventional non-uniformity of the electric field generated by multi-needle systems was solved by designing each set of three needles as an equilateral triangle.^{253a} Within the needless methods, it is worth mentioning an innovated setup that substituted the conventional spinneret with a large metal rotating cone that generated polymeric multi-jets and increased by thousands of times the traditional production rate.^{254b}

6. Conclusion

Given the complexity of biological systems, it is unlikely that mono-domain approaches would result in functional / reparative therapies. Yet again, combinatorial therapy is still at its infancy and significant limitations need addressing (**Table 5**). Proof of concept studies in the field of multi-domain / multi-cargo delivery have shown promise, however further studies are needed to demonstrate efficacy and safety and to bring these technologies to higher technology readiness level. Indeed, to-date, only a handful of complex, yet elegant, systems has been assessed in preclinical models and none of them has been brought to clinic. This limited clinical translation could be due to multi-fold restraints. The complex regulatory system concerning new molecular / regenerative medicine therapies²⁵⁶ often discourages researchers from taking innovative and possibly therapeutic concepts forward, despite the superior proof-of-concept data. Established companies are reluctant in changing their manufacturing setup, whilst, at the same time, there are not enough investment opportunities for high risk / high return concepts. Clinicians / surgeons, considering that their reputation and their patients' health or even life is at risk, are sceptical when it comes to bringing complex therapies to bedside. Patients are concerned about new therapies, especially when adequate information and data are not available. Funding opportunities for potential visionary / game-changing / disruptive technologies are limited. We can also not exclude the ever-increasing biomaterial complexity in laboratory setting that impedes industrial scalability / manufacturing; in fact, it is estimated that up to 90 % of the laboratory-based studies cannot be reproduced.²⁵⁷ It is imperative, in the years to come, researchers, clinicians / surgeons, patients, industrialists / investors, funding agencies and regulatory bodies to work together for the development of more biologically relevant, scalable, safe and regulatory-compatible multi-cargo / multi-compartment implantable devices. This would be the only way forward, the only way to make a real impact and truly advance human health.

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Figure legends

Graphical abstract: Layer-by-Layer, self-assembly, composite and core-shell technologies for simultaneous delivery of growth factors, drugs, genes and/or cells.

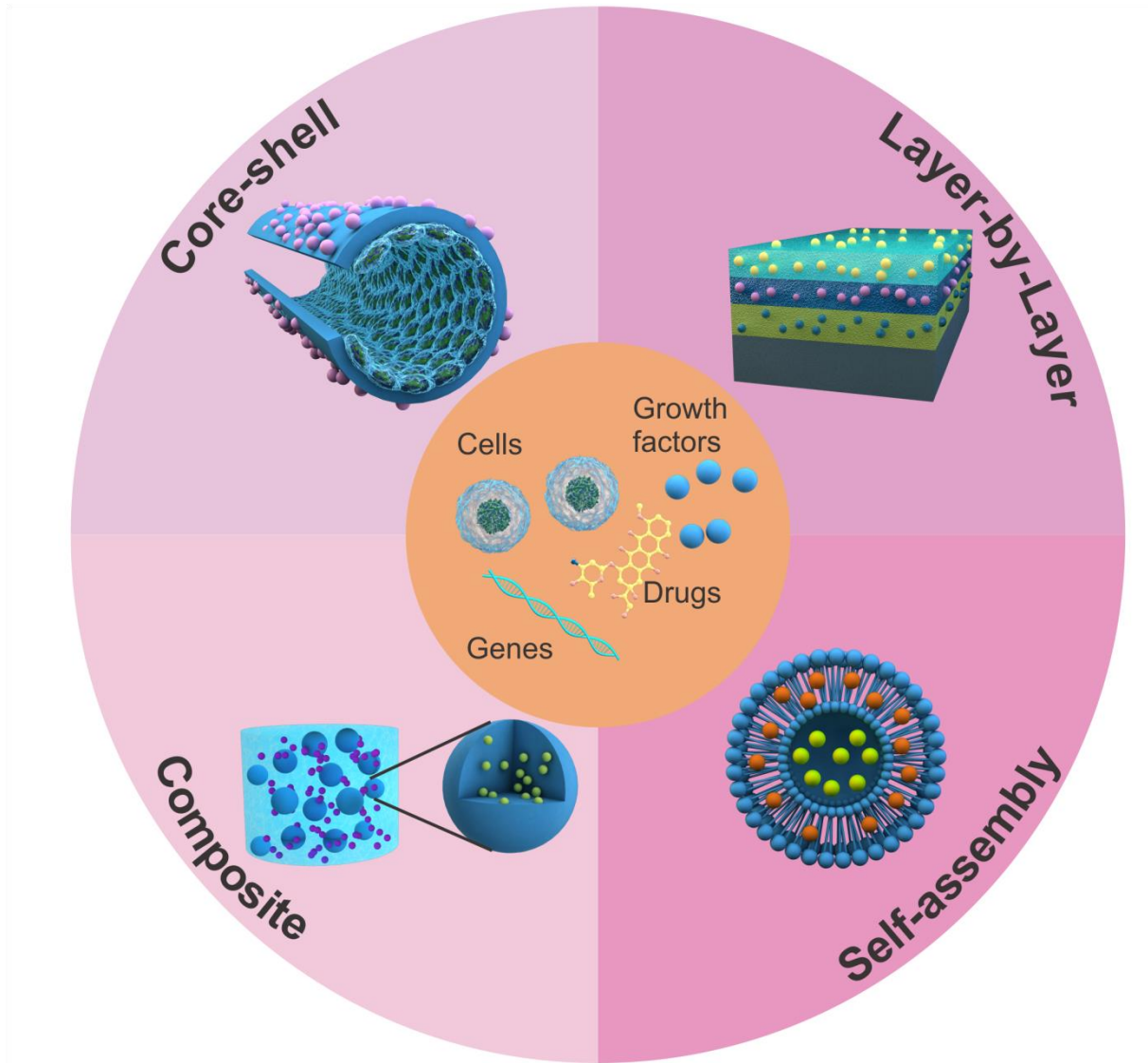


Figure 1: LbL technologies for multi-cargo delivery. Immersive, spray, spin-coating, electromagnetic and microfluidics technologies to obtain multilayered films or 3D templates (left). Indicative example of LbL technology to fabricate a 2-layer film, each loaded with different agent, onto a plane substrate (middle top). Indicative example of LbL technology to fabricate a 3D template loaded with different agents (middle bottom).

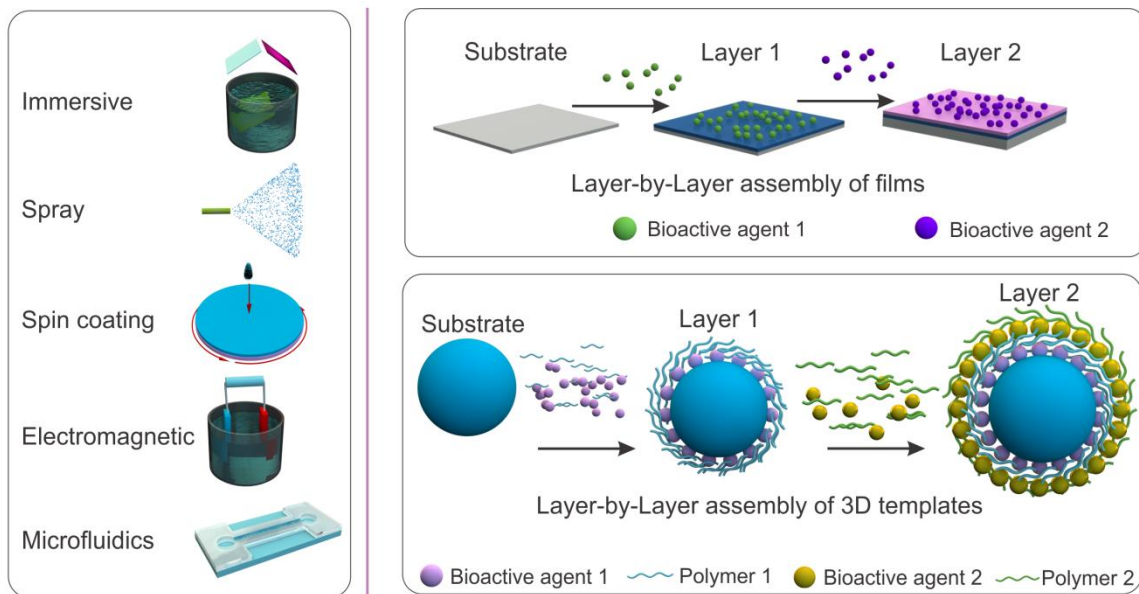


Figure 2: Self-assembly technologies for multi-cargo delivery. Spontaneous self-assembly of amphiphilic molecules coupled with encapsulation/conjugation of hydrophilic and hydrophobic bioactive agents (middle). Self-assembly of lipid, polymeric or peptidic molecules can give rise to liposomes, micelles, polymersomes or dendrimers (outer and clockwise).

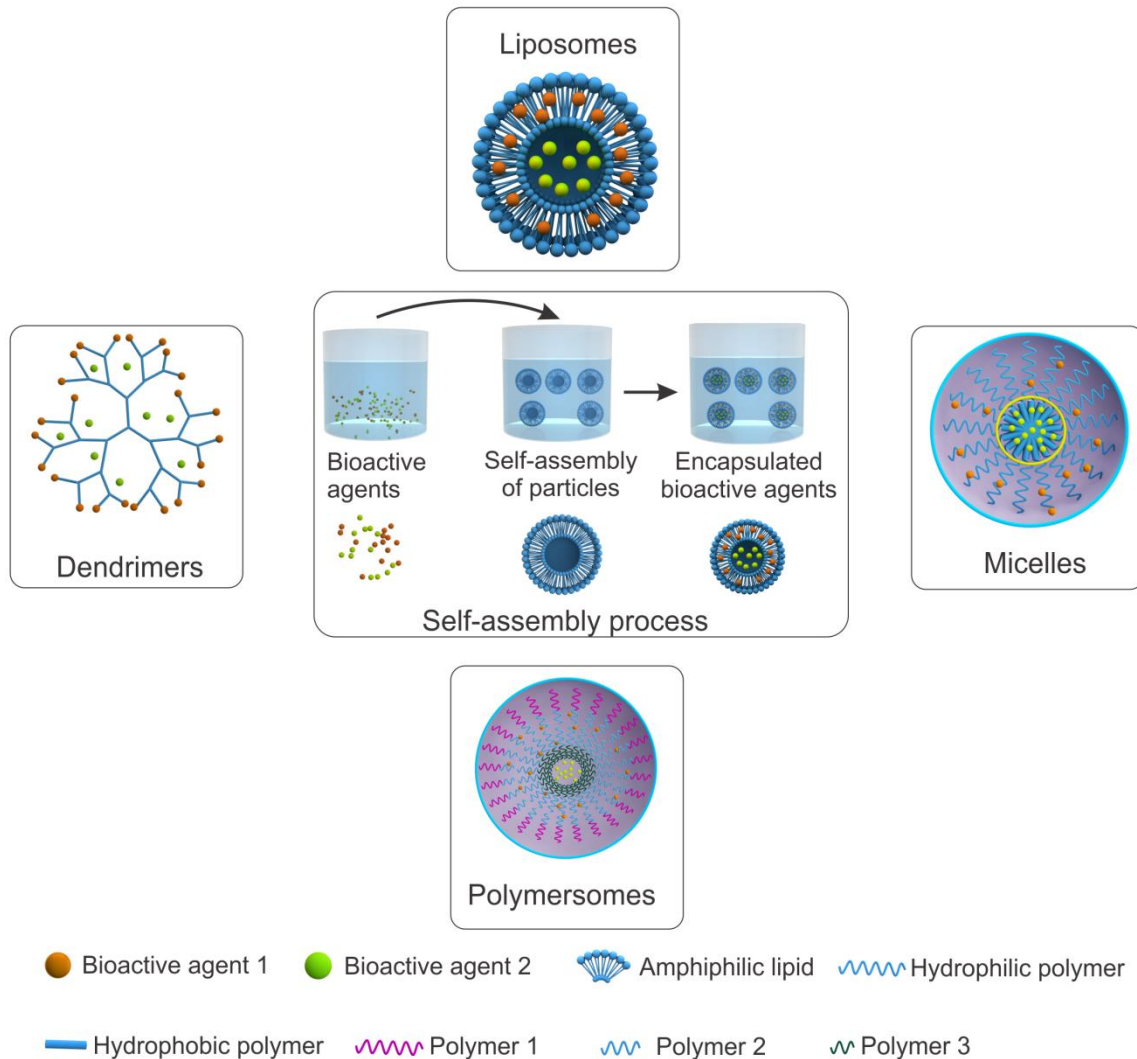


Figure 3: Composites technologies for multi-cargo delivery. Hydrogels (cargo-loaded or not) are extensively used as delivery vehicles for cargo-loaded electrospun fibers, particles and micelles (top). Porous scaffolds (e.g. sponges) and electrospun fibers are frequently used as delivery vehicles of cargo-loaded particles (bottom).

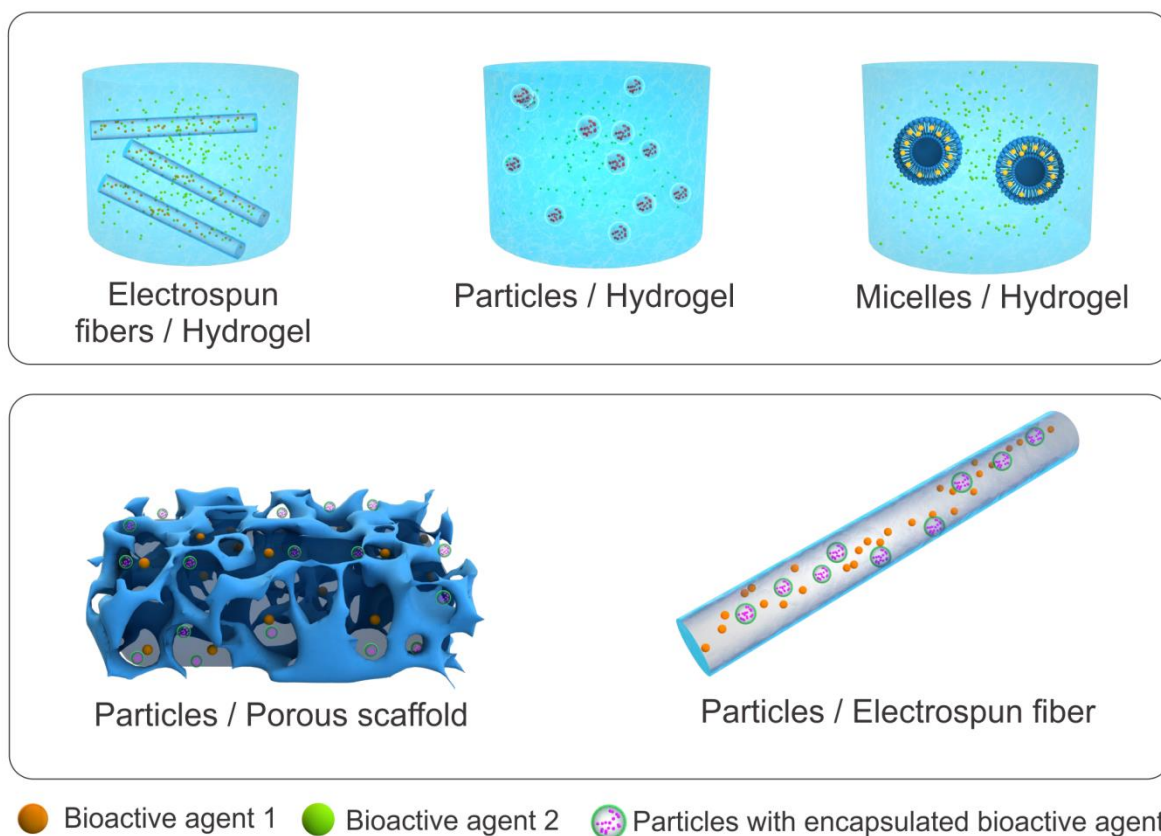


Figure 4: Core-shell technologies for multi-cargo delivery. Emulsion electrospinning (left), co-axial and tri-axial electrospinning (middle) and co-axial electrospaying are extensively used in regenerative medicine for the development of core-shell electrospun fibers and particles for the simultaneous delivery of multiple cargos.

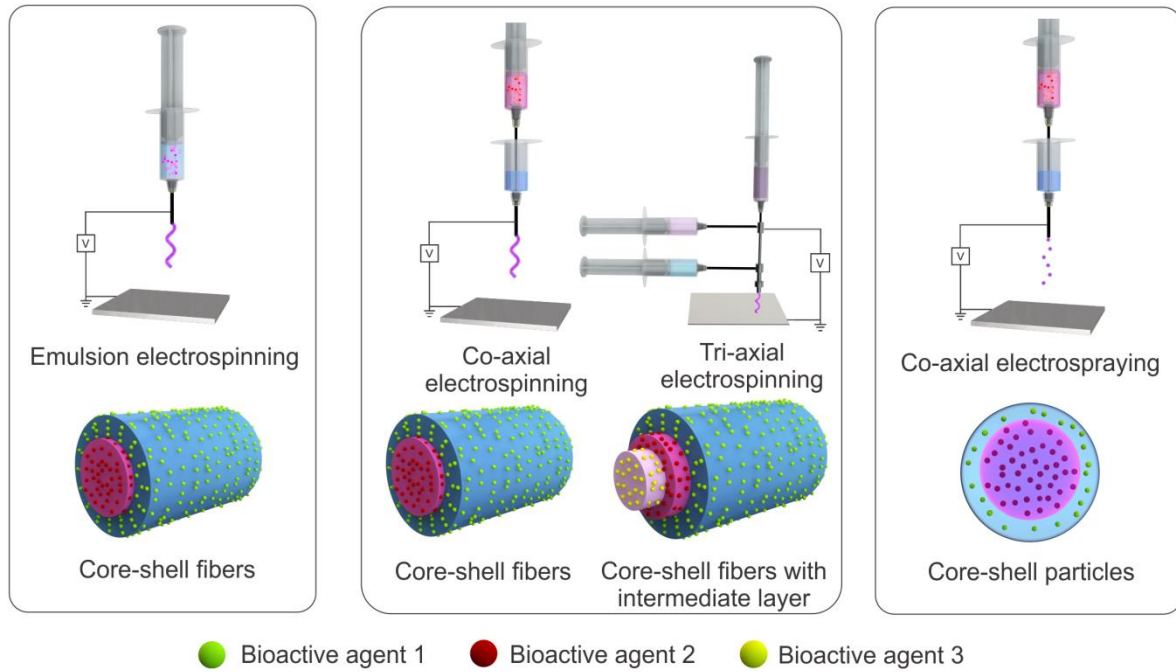


Table 1: Indicative examples of latest LbL technologies for multiple cargo delivery that have been assessed in preclinical models

Clinical target	Delivery technology	Outcome	Ref
Nucleus pulposus	Nanostructured 3D PLGA constructs loaded with DEXbFGF embedded heparin/poly(L-lysine) NPs as cell carriers (microspheres were developed in the end)	The simultaneous release of drug and GF promoted <i>in vivo</i> proliferation of rat MSCs and expression of disc-matrix proteins, whilst it decreased the expression of osteogenic differentiation marker and reduced inflammatory response	32b
Wound healing	Assembly of HA and CHI on the internal surfaces of porous PLGA microspheres layered with α -MSH, followed by bFGF loading assembled by exploiting electrostatic interactions	The system preserved the bioactivity of α -MSH and reduced inflammatory response <i>in vivo</i> , compared to the non-layered microspheres; no benefit was observed by the co-delivery of bFGF	258
	Multilayer films of CHI and ALG containing EGF and TGF- β siRNA assembled by exploiting electrostatic interactions	The co-delivery of TGF- β siRNA and EGF resulted in greater re-epithelialization, accelerated wound healing and decreased scar formation <i>in vivo</i> , as compared to the single delivery	36c
Cartilage	Embedment of DHEA-PLGA negatively charged microspheres into DEX-PLGA positively charged microspheres followed by MSCs entrapment	The system sustained the simultaneous delivery of drugs and cells <i>in vivo</i>	49

Bone	Tetra-layered films assembled by exploiting electrostatic interactions between layers of POLY-2, PAA, rhBMP-2 or rhVEGF165 and again PAA	The controlled release of rhVEGF165 (placed on the top layer) and rhBMP-2 (placed on the bottom) enabled blood vessel formation and new bone formation	32a
Anti-cancer	Multilayer micro-dispersing system constructed by co-immobilization of DOX-loaded CHI / CM-CHI nano-gels into QU-immobilized multilayer ALG beads	The co-delivery <i>in vivo</i> of an anticancer drug and QU (P-gp inhibitor) improved DOX bioavailability, oral absorption and targeted release in the small intestine	36b
	DTX-encapsulated BSA-PEI-NPs assembled by exploiting electrostatic interactions between PEI, p44/42 MAPK siRNA and the antigen PSMA	The co-delivery of an anticancer drug and siRNA overcame MDR and selectively inhibited <i>in vivo</i> prostate cancer cells proliferation by conjugation with PSMA	32c

Abbreviations: ALG: alginate; α -MSH: α -melanocyte-stimulating hormone; bFGF:basic fibroblast growth factor; BSA: bovine serum albumin; CHI: chitosan; CM-CHI: carboxymethyl chitosan; DEX: dexamethasone; DHEA: dehydroepiandrosterone; DTX: docetaxel; EGF:epithelial growth factor; HA: hyaluronic acid; HEP: heparin; MSC: mesenchymal stem cells; P44/42 MAPK: P44/42 mitogen-activated protein kinase; PAA: polyacrylic acid; PEI: polyethyleneimine; PLGA: poly(L-lactic-co-glycolic) acid; POLY-2: Poly (β -aminoester) 2; PSMA: prostate specific membrane antigen; QU: quercin; rhBMP-2: recombinant human bone morphogenetic protein-2; rhVEGF165: recombinant human vascular endothelial growth factor 165; TGF- β : transforming growth factor β

Table2: Indicative examples of latest self-assembly technologies for multiple cargo delivery that have been assessed in preclinical models

Technology	Clinical target	Delivery technology	Outcome	Ref
Liposomes	Anti-cancer	Encapsulation of QCT and RES into NPs using the thin film formation technique and followed by rehydration and ultra-sonication	The system co-delivered both cargos, increased cellular uptake and reduced edema and leukocyte infiltration <i>in vivo</i>	259
		Encapsulation of pEGFP-hTRAIL and PTX, into thin film ANG functionalized cationic NPs	The system co-delivered hTRAIL and PTX, resulting in increased uptake and gene expression in glioma mice models <i>in vivo</i> , increasing selective cytotoxicity to tumor cells and animal median survival time	78
		Encapsulation of DOX and P-gp inhibitor into reduction-sensitive PEG/R8 conjugated thin film NPs	The system inhibited tumor growth <i>in vivo</i> and reduced the systemic toxicity of DOX and the P-gp inhibitor	260
		Encapsulation of DOX and PTX into thin film DTT cross-linked multilamellar liposomal vesicle	The system maintained specific drug ratios <i>in vivo</i> in BALB/c mice inoculated with 4TI tumors cells, after 48 h and 24h of administration, respectively.	98

Polymersomes	Anti-cancer	Encapsulation of DOX and TET into LF conjugated methoxy PEG-PCL and α -carboxyl PEG-PCL block NPs	The system crossed the BBB and accumulated preferentially <i>in vivo</i> at the glioma site and promoted a higher tumor reduction than single formulations	131c
PAMAM dendrimers	Anti-cancer	Encapsulation of DOX and MMP-9 shRNA into a functionalized pH responsive GO-PAMAM NPs	The system enabled pH dependent DOX release at weak acidic conditions and was less cytotoxic to normal tissues <i>in vivo</i>	140b
		Encapsulation of DDP and siRNA into Selenium functionalized PAMAM NPs	The system showed reduced systemic cytotoxicity and selective toxicity in A549/DDP lung cancer cells <i>in vitro</i> and enhanced the anti-tumor effect in tumor-bearing nude mice <i>in vivo</i>	132e
		Encapsulation of DOX and pORF-hTRAIL into T7 modified PEG-PAMAM NPs	The system promoted accumulation, apoptosis and tumor reduction in tumor cells <i>in vivo</i> more efficiently than unmodified or single cargo formulations	132b
Micelles	Anti-cancer	Encapsulation of DOX and TPGS1000 into pH-responsive PEOz-PLA-DSPE-PEG-FA NPs obtained through ring-opening polymerization	The system promoted specific pH triggered release and accumulation in tumor tissues <i>in vivo</i> , when compared to other normal tissues	146b

		Encapsulation of cRGDyK and DOX into amphiphilic PEG-PLA copolymer NPs	The system provided an optimal antitumor effect <i>in vivo</i> , promoted lifespan increase, anti-neovasculature and anti-proliferation	261
Micelles	Anti-cancer	Encapsulation of DOX and PTX into Pluronic P105 and F127 copolymer NPs	The system provided a stronger synergistic antitumor effect <i>in vivo</i> , when compared to single formulations of DOX and PTX	262
		Encapsulation of MMC and MTX into DSPE-PEG copolymer NPs	The system improved tumor accumulation, penetration and anticancer effect <i>in vivo</i> , when compared to single formulations	263
		Encapsulation of PTX, RAP and 17-AAG into PEG-PLA copolymer NPs	The system induced tumor regression <i>in vivo</i> after four weeks, as opposed to single formulations, and did not induce any changes in body weight	264
		Encapsulation of PTX and siRNA into pH and reduction sensitive LDL-NSC-LA NPs	The system allowed cancer cell targeting, induced tumor inhibition <i>in vivo</i> and showed decreased systemic toxicity, when compared to single formulations	147
		Encapsulation of siRNA and PTX into triblock PEG-PCL-PAEP NPs	The system promoted a synergistic effect leading to increased tumor suppression <i>in vivo</i> in murine model, when compared to single formulations, without associated cytotoxicity	265

		Encapsulation of siRNA and DOX into pH sensitive triblock PEG-PAsp(AED)-PDPA NPs	The system promoted increased apoptosis of human ovarian cancer cells and inhibited tumor growth <i>in vivo</i>	266
Micelles	Anti-cancer	Encapsulation of mcDNA and DOX into triblock bioreducible PEOz-PLA-PEI NPs	The system induced tumor volume reduction and decreased cancer cell viability <i>in vivo</i>	267
		Encapsulation of AXI and DOX into shell cross-linked NPs of HPMA	The system improved tumor accumulation, reduced the number of immature vessels and inhibited tumor growth, when compared to single formulations	268
		Encapsulation of CPT and TNF plasmids into star-shaped copolymer NPs of pH and redox-responsive bPEG-PAsp(DET)	The system enhanced gene transfection and cytotoxicity in 4T1 cells and in tumor models, when compared to single formulations	269
	Vaccine	Encapsulation of OVA and CpG ODN adjuvants into NPs doped with thiol reactive groups	The system increased antigen cross-presentation, T-cell response and immunization after vaccination of mice, when compared to single formulations	270

Abbreviations: 17-AAG: 17-allylamino-17-demethoxygeldana-mycin; ANG: angiopep-2; AXI: axitinib; BBB: blood brain barrier; CpG ODN: immunostimulatory CpG oligodeoxynucleotide; CPT: Camptothecin; cRGDyK: cyclic arginine-glycine-aspartic acid-tyrosine-lysine pentapeptide; DDP: cisplatin (cis-diamminedichloroplatinum-(II)); DSPE-PEG: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000];

DTT: dithiothreitol; DTX: docetaxel; FA: folate; GO: graphene oxide; hTRAIL: human tumor necrosis factor-related apoptosis-inducing ligand; HPMa: N-(2-hydroxypropyl) methacrylamide; LA: lipoic acid; LDL: low density lipoprotein; LF: lactoferrin; mcDNA: minicircle DNA; MMC: methotrexate; MTX: methotrexate; NP: nanoparticle; NSC: N-succinyl chitosan; OVA: ovalbumin; PAEP: poly(2-aminoethyl ethylene phosphate); PAsp(AED): poly(N-(2,2'-dithiobis(ethylamine)) aspartamide); PAsp(DET): poly(N-(2,2'-dithiobis(ethylamine)) aspartamide)-Diethylenetriamine; PAMAM: poly(amidoamine); PCL: poly-(caprolactone); PDPA: 2-(diisopropylamino)ethyl methacrylate; PEG: poly-(ethylene glycol); PEI: polyethyleneimine; PEOz: poly(2-ethyl-2-oxazoline); P-gp inhibitor: P-glycoprotein inhibitor; PLA: poly-(lactic acid); pORF-hTRAIL – therapeutic gene for human tumor necrosis factor-related apoptosis-inducing ligand; PTX: paclitaxel; QCT: quercetin; R8: octaarginine; RA: retinoic acid; RAP: rapamycin; RES: Resveratrol; shRNA: short hairpin RNA; siRNA: small interfering RNA; T7: transferrin receptor-specific peptide; TET: tetracycline; TNF: Tumor necrosis factor; TPGS: D-alpha-tocopheryl poly (ethylene glycol 1000) succinate

Table 3: Indicative examples of latest composite technologies for multiple cargo delivery that have been assessed in preclinical models

Clinical target	Delivery technology	Outcome	Ref
Angiogenesis	DEX and VEGF-loaded PLGA microsphere / PVA hydrogel composites prepared by dispersing microspheres into the PVA solution	The co-delivery of the drug and the GF minimized inflammation, inhibited fibrosis and promoted neo-angiogenesis at the implant site	271
	IPC fibers incorporated into PVA hydrogel loaded with VEGF and PEG-QK peptide	The <i>in vivo</i> implantation of PVA–IPC composite grafts had mechanical properties matching rabbit femoral artery and showed potential as vascular graft up to 28 days	185
	Delivery platform for VEGF and TGF-β3 comprised of coacervate (PEAD polycation, HEP, and GFs) - coated nano-fibrous PLGA membrane	The co-delivery of VEGF and TGF-β3 reduced necrosis and enhanced blood perfusion at the implanted area <i>in vivo</i>	272
Bone	VEGF and BMP-2-loaded GEL microparticles confined within a PPF porous scaffold	The dual release of VEGF and BMP-2 showed the complete union of the bone defect <i>in vivo</i> and no visible effect on blood vessels formation	191
	PLGA microspheres loaded with BMP-2 embedded in a PP scaffold surrounded by a GEL hydrogel loaded with VEGF	The combined angiogenic and osteogenic GFs release <i>in vivo</i> enhanced bone regeneration in the ectopic implantation site	273

	A brushite- CHI system with VEGF-loaded ALG microsphere-encapsulated into PDGF-loaded CHI sponges	The controlled <i>in vivo</i> release of both GFs within a bone defect resulted in greater bone formation than the single GF delivery	166
	IGF-1 and TGF -loaded GEL microcapsules encapsulated into a bi-layered OPF hydrogel fabricated by cross-linking procedure	The single delivery of IGF-1 resulted in greater cartilage repair when compared to a dual delivery of IGF-1 and TGF- β 3, suggesting lack of synergy between the GFs	274
	VEGF and BMP2 – loaded nHA particles combined with highly porous cross-linked COLL-based scaffold	The composite increased MSC-mediated osteogenesis <i>in vitro</i> and vascularization and bone repair by host cells <i>in vivo</i>	275
Anti-cancer	PTX – loaded PEG-PCL micelles mixed with DDP-loaded PEG-PCL-PEG hydrogel	The thermosensitive and injectable composite significantly inhibited lung tumor growth <i>in vivo</i> and angiogenesis in tumor tissue	181b
	PTX-loaded PECT NPs embedded into DOX PECT hydrogel	An initial burst release of DOX improved the <i>in vivo</i> anti-tumor effect, which was further increased by the subsequent sustained release of PTX-incorporated nNPs	276
	DOX and I ¹³¹ -HA – loaded thermosensitive injectable PECT hydrogel / micelles composite	The composite showed <i>in vitro</i> antitumor effect and radiosensitization and improved the tumor growth inhibition efficiency <i>in vivo</i> with minimized drug-associated side effects	181a

	PTX-loaded PCEC micelles mixed with FU - loaded thermosensitive PCEC hydrogel	The <i>in vivo</i> implantation of PTX-micelles–Fu-hydrogel inhibited growth and metastasis of peritoneal carcinomatosis	181c
	PTX-loaded NPs and LAPA-loaded microparticles into thermosensitive PLU F127 hydrogel	The composite enabled short-term <i>in vivo</i> release of PTX and long-term release of LAPA for a synergistic effect against breast cancer	132e
	DOX-loaded Au / BSA NPs assembled through electrostatic interactions on GEM-loaded mesoporous Si-NPs	The system efficiently delivered both drugs <i>in vivo</i> to cancer cells with a nearly total cell killing	277
	TET/PTX-loaded PEG-b-PCL NPs encapsulated within a thermo-responsive GEL hydrogel	The composite inhibited the growth, migration and invasion of cancerous gastric cells more effectively than drugs-loaded NPs	278
Platform	PLGA/PCL electrospun fibers loaded with DPV, MVC and TFV encapsulated into pHEMA hydrogel	The hydrogel-tissue mimic established <i>in vitro–ex vivo</i> correlations with topical and transdermal drug delivery into vaginal mucosal tissue explants	279

<p>Wound healing</p>	<p>PDGF and VEGF loaded-GEL NPs embedded into bFGF and EGF loaded-HA/COLL electrospun-fibers</p>	<p>The early stage <i>in vivo</i> delivery of EGF and bFGF accelerated epithelialization, whilst the later release of PDGF and VEGF allowed induction of blood vessels maturation</p>	<p>167b</p>
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Abbreviations: ALG: alginate; Au: gold; BMP-2: bone morphogenetic protein-2; BSA: bovine serum albumin; CHI: chitosan; COLL: collagen; DDP: cisplatin (cis-diamminedichloroplatinum-(II)); DEX: dexamethasone; DOX: doxorubicin; DPV: dapivirine; FU: fluorouracil; GEL: gelatin; GEM: gemcitabine; HEP: heparin; HGF: hepatocyte growth factor; I¹³¹: iodine¹³¹; IGF-1: insulin-like growth factor – 1; IPC: interfacial polyelectrolyte complexation; LAPA: lapatinib; MVC: maraviroc; nHA: nano hydroxyapatite; OPF: oligo (poly (ethylene glycol) fumarate); PCEC: poly-(3-caprolactone)–poly (ethylene glycol)–poly(3-caprolactone); PCL: poly-(caprolactone); PDGF: platelet derived growth factor; PEAD: poly (ethylene arginanyl aspartate diglyceride); PECT: poly (ε-caprolactone-co-1,4,8-trioxa [4.6] spiro-9-undecanone)-poly(ethyleneglycol)-poly (ε-caprolactone-co-1,4,8-trioxa [4.6] spiro-9-undecanone); PEG: poly- (ethylene glycol); PEG-QK: PEGylated-QK peptide; pHEMA: poly(2-hydroxyethyl methacrylate); PLGA: poly(L-lactic-co-glycolic) acid; PLU F127: pluronic; PP: polypropylene; PPF: poly (propylene fumarate); PTX: paclitaxel; PVA: polyvinyl alcohol; Si: Silica; TET: tetrandine; TFV: tenofovir; TGF: transforming growth factor; VEGF: vascular endothelial growth factor

Table 4: Indicative examples of latest core-shell technologies for multiple cargo delivery that have been assessed in preclinical models

Clinical target	Delivery technology	Outcome	Ref
Wound healing	Nano-fibrous membrane with HA as core and Ag NPs-embedded PCL as shell fabricated by co-axial electrospinning	The controlled <i>in vivo</i> release of HA from the core mimicked the biological function of HA in synovial fluid of tendons, whilst the shell acted as a physical barrier with antimicrobial activity	167c
	Nano-fibrous scaffold of HA/PCL as shell and EGF/BSA as core formed by emulsion electrospinning	The co-delivery of EGF and HA up-regulated collagen and TGF-b1 gene expression <i>in vivo</i> and stimulates epidermis regeneration in the early phases of wound healing	216
Angiogenesis	CHI hydrogel / PELCL electrospun membrane loaded with VEGF as inner layer and emulsion / PELCL electrospun membrane-loaded PDGF as the outer	The <i>in vivo</i> rapid release of VEGF ensured at first epithelization by vascular endothelial cells, followed by a sustained release of PDGF, which led to proliferation of vascular smooth muscle cells in the later time	233
	Electrospun-fibers with encapsulated pDNACa Phos NPs encoding VEGF and bFGF	The localized and sustained release of pDNA facilitated the proliferation, gene transfection and ECM secretion of	167a

		endothelial cells and smooth muscle cells and enhanced the <i>in vivo</i> angiogenesis	
	Co-axial electrospun fibers composed by a core of VEGFA/PDGF or VEGFC and a shell layer made of PU	The system provided sustained co-delivery of the GFs, stimulated the vascular system and reduced blood coagulation in a hemophilic mouse model, whilst a single GF delivery induced leaky and non-functional blood vessels	220
Bone	Hydrogel scaffold comprised of BMP-2-loaded COLL-based core and Co ions-loaded ALG-based shell	The rapid release of Co ions led to up-regulation of angiogenic genes, whilst the slower release of BMP2 led to higher expression of osteogenic genes, allowing <i>in vivo</i> enhanced bone formation	280
	Simvastatin-loaded-core and PDGF-loaded-shell PDLLA-PLGA microspheres	The fast-release of PDGF followed by a slower release of simvastatin accelerated <i>in vivo</i> osteogenesis, bone maturation, fibers re-alignment, and cemento-genesis of the periodontal apparatus	281

	BMP-2-loaded BSA NPs encapsulated within DEX-loaded PCL/PEG co-electrospun fibers	An earlier controlled release of DEX promoted <i>in vivo</i> bone formation, whereas BMP-2 release was beneficial for long-term new bone formation	188
Anti-cancer	Nano-carrier platform for co-delivery of PTX (in the shell) and DOX (in the core) based on a water-in-oil-in-water core-shell PVA nano-capsules	The system allowed on-demand drug release <i>in vitro</i> and <i>in vivo</i> using a magnetic field trigger and addressed tumor cells thanks to the conjugation of a tumor-targeting peptide to the nano-capsules, achieving the complete suppression of the tumor	282
	NPs of DTX physically entrapped into PBS/PBDL copolyester (as core) and DOX covalently attached to the HPMA-based copolymer (as shell)	The system allowed high drug encapsulation, DOX pH-triggered drug release and <i>in vivo</i> reduction of tumor growth	283
	Electrospun-PLGA microfibers encapsulating PTX and PEI/DNA NPs	Gene/drug dual delivery microfibers imposed tumor regression <i>in vivo</i> , as compared with single drug delivery microfibers	284
	Lf-tethered magnetic double emulsion nano-capsules assembled from PVA, PAA and IO NPs can accommodate simultaneously DOX and CUR, in the core and shell, respectively	The release patterns of the two drugs was regulated by manipulating the surface charges and drug-loading ratios and the nano-capsule was effectively delivered <i>in vivo</i> into RG2 glioma cells thanks to LF tag	146a

<p>Platforms</p>	<p>A cell-containing ECM-protein/Ca-ALG core-shell hydrogel microfiber generated in a double-coaxial microfluidic device and encapsulating multiple types of cells</p>	<p>The meter-long fiber-like structures showed very similar tissue functionality. Fibers encapsulating primary pancreatic islet cells and transplanted IN diabetic mice normalized blood glucose concentrations <i>in vivo</i></p>	<p>239</p>
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Abbreviations: Ag: silver; ALG: alginate; bFGF: basic fibroblast growth factor; BMP-2: bone morphogenetic protein 2; BSA: bovine serum albumin; Ca: calcium; CHI: chitosan; Co: cobalt; COLL: collagen; CUR: curcumin; DOX: doxorubicin; DTX: docetaxel; EGF: epithelial growth factor; GEL: gelatin; HA: hyaluronic acid; HPMA: N-(2-hydroxypropyl) methacrylamide; IO: iron oxide; Lf: lactoferrin; PAA: polyacrylic acid; PBS/PBDL: (poly (butylene succinate-co-butylene dilinoleate)); PCL: poly-(caprolactone); PDLLA: poly(d,L)lactic acid; PDGF: platelet derived growth factor; PEI: polyethyleneimine; PELCL: poly (ethylene glycol)-b-poly (L-lactide-co-capro- lactone); PGA: *poly(glycolic acid)*; Phos: phosphate; PTX: paclitaxel; PU: poly(urethane); PVA: polyvinyl alcohol; VEGF: vascular endothelial growth factor

Table 5: Advantages and challenges of multi-cargo delivery technologies

Technology	Advantages	Challenges
LbL technology	<ul style="list-style-type: none"> ▪ surface functionalization for active targeting ▪ coatings performed at physiological conditions ▪ automated production techniques available 	<ul style="list-style-type: none"> ▪ interlayer diffusion ▪ stability and fate poorly understood ▪ polyelectrolytes cytotoxicity ▪ loading of the cargo limited by the molecular interactions
Self-assembly technology	<ul style="list-style-type: none"> ▪ incorporation of multiple cargos with different solubility into distinct layers ▪ controllable polydispersity and size distribution ▪ surface functionalization for active targeting and reduced immunoreactivity ▪ low cytotoxicity ▪ scalability 	<ul style="list-style-type: none"> ▪ poor loading capabilities ▪ dendrimers cytotoxicity
Composite technology	<ul style="list-style-type: none"> ▪ superior physicochemical and biological properties than single material 	<ul style="list-style-type: none"> ▪ technically complex optimization of the mixtures ▪ difficulty of characterization, toxicity assessment and scalability

Core-shell technology	<ul style="list-style-type: none">▪ surface functionalization for active targeting on particles▪ possibility to incorporate bioactive agents into a core made of fluids▪ reproducibility	<ul style="list-style-type: none">▪ multi-step surface functionalization for fibers▪ use of emulsified agent in emulsion electrospinning▪ slow production rate
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Authors bibliography

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