

Advances on Graphene-Based Nanomaterials and Mesenchymal Stem Cell-Derived Exosomes Applied in Cutaneous Wound Healing

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Abstract: Graphene is a new type of carbon nanomaterial discovered after fullerene and carbon nanotube. Due to the excellent biological properties such as biocompatibility, cell proliferation stimulating, and antibacterial properties, graphene and its derivatives have become emerging candidates for the development of novel cutaneous wound dressings and composite scaffolds. On the other hand, pre-clinical research on exosomes derived from mesenchymal stem cells (MSC-Exos) has been intensified for cell-free treatment in wound healing and cutaneous regeneration, via ameliorating the damaged microenvironment of the wound site. Here, we provide a comprehensive understanding of the latest studies and observations on the various effects of graphene-based nanomaterials (GBNs) and MSC-Exos during the cutaneous wound repair process, as well as the putative mechanisms thereof. In addition, we propose the possible forward directions of GBNs and MSC-Exos applications, expecting to promote the clinical transformation.

Keywords: graphene, carbon nanomaterials, mesenchymal stem cells, exosomes, wound healing

Introduction

Graphene-based nanomaterials (GBNs), as a group of next-generation nanomaterials, have offered tremendous potential in a diverse range of applications.¹ Since firstly prepared by mechanical exfoliation in 2004,² graphene has been widely used in flexible supercapacitors, electrochemical sensors and batteries, etc.³⁻⁶ In recent years, due to its excellent electronic, thermal and optical properties, graphene has attracted enormous interest.⁷⁻⁹ The graphene plasmons could couple with other plasmons and phonon polaritons, leading to a further mediation of near-field radiative heat transfer.¹⁰ Moreover, graphene is expected to become an ideal membrane material to separate gases, liquids and ions in terms of the selectivity and permeability, which could outperform the established polymer membranes.^{11,12}

Likewise, a growing number of studies have also focused on the application of graphene and its derivatives in the field of biomedicine, which is in full swing and involves drug carriers, biological detection, tumor treatment and tissue engineering.¹³ Investigations on the 2D or 3D scaffold for cell culture suggest the ability of GBNs for mimicking in vivo environment.

Under normal circumstances, cutaneous wound healing is a rapid and efficient process leading to the reconstruction of barrier function.¹⁴ But the repair process

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may be delayed by systemic or local factors, incurring significant costs and loss of life quality to the patients involved.^{15,16} Various types of novel bioactive skin substitutes and cell-based therapies have emerged continually in pre-clinical and clinical studies, providing promising ideas for the repair of large-area or difficult-to-heal wounds.¹⁷ All prokaryotes and eukaryotes are able to release extracellular vesicles (EVs) under physiological or pathological conditions. Exosomes are a subset of EVs that originate from the endosome, the diameter of which ranges from 40 to 160 nm (average 100 nm).¹⁸ The diverse constituents generally include protein, miRNA, mRNA, DNA and lipid, and participate in intercellular communication.^{19,20} Studies have found that the application of exosomes, especially which come from mesenchymal stem cells (MSCs), can effectively repair the cutaneous wound and achieve the promising healing.²¹

In this review, we focus on the booming application of graphene-based materials and MSC-Exos in cutaneous wound repair. Besides, the structure and synthesis methods of GBNs are briefly summarized, and then we highlight their excellent biological properties and biocompatibility. Finally, we present our perspectives on the potent synergistic effect between GBNs and MSC-Exos for the treatment of cutaneous wound. We also put forward the potential challenges and opportunities facing in future research.

Cutaneous Wound Healing

The cutaneous wound healing process is divided into four overlapping phases, defined as hemostasis, inflammation, proliferation and tissue matrix remodeling.²² Dysregulation of any stage would increase the risk of chronic refractory cutaneous wounds, with growing morbidity and mortality.²³ After the skin is injured, local tissue initiates necrosis and bleeding. Next, blood vessels constrict and blood clots form, providing a basic environment for the subsequent wound healing.²⁴ The blood clots can attract platelets, growth factors, cytokines, neutrophils, macrophages and endothelial cells, which collaborate well to form blood scabs so as to protect the wound, until the blood vessels dilate.²⁵ Coagulation factors play a vital role in the infiltration of relevant cells, typically the macrophages. The mice of the hemophilia B model exhibit an impaired healing of skins, articular cartilages and bones.²⁶ The hemostatic protein von Willebrand factor (VWF) released by endothelial cells has been shown to regulate angiogenesis, and VWF deficiency in mice causes

delayed wound healing as well as decreased angiogenesis and decreased amounts of angiogenic growth factors in the wound.²⁷

Cutaneous injury leads to the prompt activation of a topical inflammatory response. Moderate inflammation is a highly regulated process orchestrated by resident cells and immune cells recruited spatiotemporally, fighting against infection and removing dead cells and cell debris^{28,29} (Figure 1). On contrary, immoderate inflammation causes prolonged wound healing. Several kinds of innate immune cells play a pivotal role in this stage.³⁰ The proper termination of inflammatory cascade is important to allow the execution of tissue repair and restoration of normal tissue function. Neutrophils are the first responders to epithelial injury. They infiltrate into wound tissue, clear bacteria, limit infection, and secrete proinflammatory TNF- α , which stimulates fibroblast proliferation and angiogenesis.^{28,31} Landén et al reviewed that Omega-6 Fatty Acids presented the capacity to improve wound healing due to antioxidant and anti-inflammatory effects on the inflammatory phase of tissue repair.³²

Three to ten days after the wound, the healing program enters the phase of proliferation. In this phase, re-epithelialization and angiogenesis overlap leading to the formation of healthy and vascularized granulation tissue. Owing to ischemia and hypoxia, the proliferation and migration capacity of dermal fibroblasts decreased and incurred refractory wounds. Transition from inflammation to proliferation is a critical step during wound healing. Factors that impact this phase transition involved macrophages, redox signals and TLR signaling.³² Li et al³³ proved that inhibition of miR-132 in keratinocytes may delay the transition from the inflammatory to the proliferative phase during wound healing. Single-cell RNA-sequencing analysis distinguished differential subpopulations of dermal fibroblasts³⁴ with heterogenous function.³⁵ The shift in fibroblast composition influenced wound healing rate in vivo. TNF signaling and several other intrinsic and extrinsic cytokines seemed key contributing factors to the variability in ageing phenotypes, including wound healing, and even detrimental fibrosis in vivo.³⁴

Extracellular matrix remodeling and scar formation are the final stages of wound closure. It begins 2 to 3 weeks following the initial injury and lasts up to a year or more, depending on wound severity. The imbalance in synthesis and degradation of extracellular matrix may lead to wound non-healing or pathological scar formation.^{36,37} The pro-inflammatory cytokine TNF- α enhances collagenolysis and increases the activation of MMP-13 by up-regulating MMP-3.³⁸ Compared to the

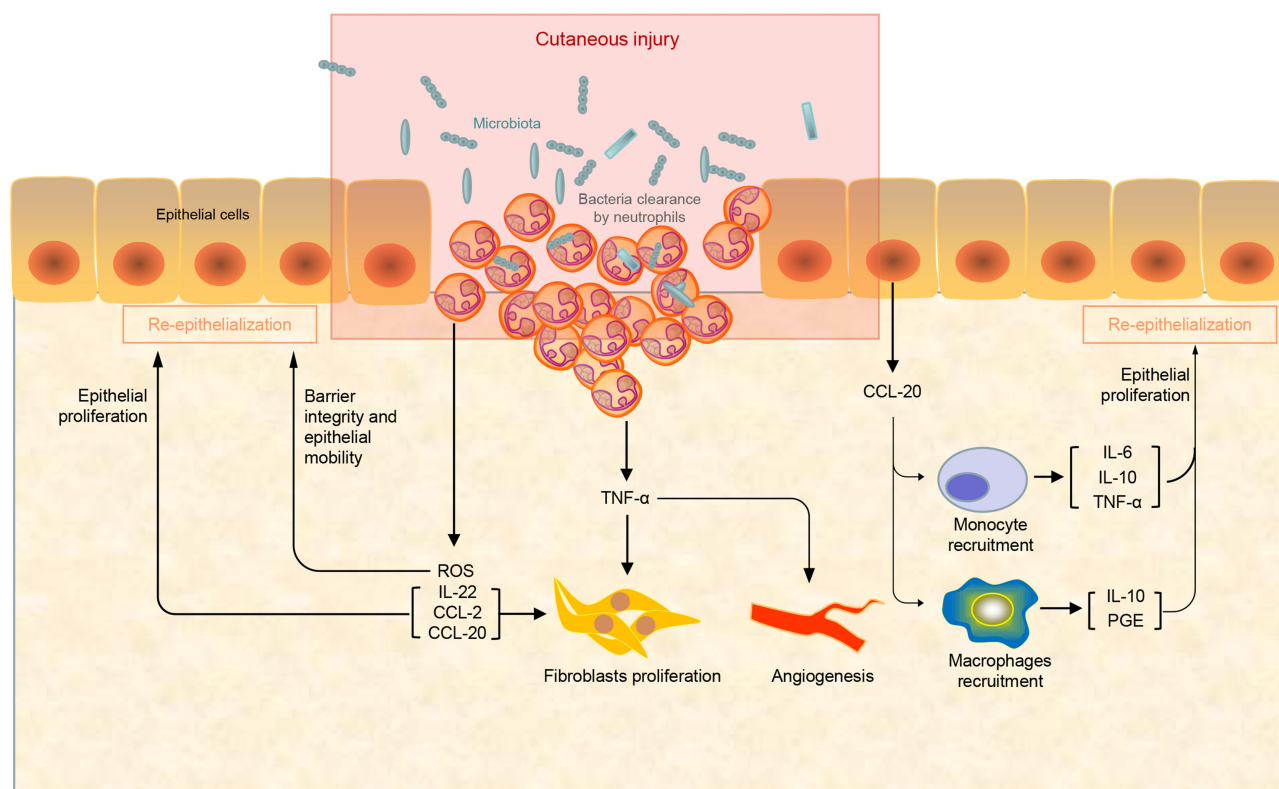


Figure 1 The proinflammatory stage of wound healing. Neutrophils respond quickly once the cutaneous injury happened to limit bacterial infections at the wound site and stimulates fibroblast proliferation and angiogenesis by the secretion of series of cytokines.

physiologic scarring in adults, fetal cutaneous wound healing is uniquely characterized by repair with complete restoration of dermal architecture. Microarray analysis revealed that signaling pathways, like proline biosynthesis I, IL-8, CXCR4, Neurotrophin/TRK, were differentially expressed in E17 fetal versus adult wounds.³⁹

Graphene and Graphene-Based Nanomaterials

Structure and Synthesis

Graphene is an allotrope of carbon. Briefly, it is a two-dimensional hexagonal lattice with a flat monolayer of carbon atoms formed by strong triangular bonds in sp^2 hybrid orbitals, forming the basis of both 3D graphite and 1D carbon nanotubes.^{40,41} Graphene oxide (GO) is obtained by multi-step oxidation of graphene and ultrasonic purification. It contains hydroxyl, epoxy and carboxyl groups on the edge of the sheet, effectively increasing the interlayer spacing.⁴² Reduced graphene oxide (rGO) is produced from GO through a variety of reduction processes.⁴³ To fully exploit the properties of graphene and its derivatives for biomedicine applications, it requires

an ideal method for the mass production of this remarkable material.⁴⁴ This remains a major challenge. So far, a number of techniques have been developed including mechanical cleavage, liquid-phase exfoliation and chemical vapor deposition, etc.

The graphite is composed of graphene sheets held together by Van der Waals interactions, therefore, graphene sheets can be torn down from graphite by applying mechanical force.⁴⁵ Meyer et al prepared monolayer graphene flakes on top of an oxidized silicon wafer by mechanical cleavage.⁴⁶ Analogously, liquid-phase exfoliation is defined that the graphite is dispersed and exfoliated in organic solvents.⁴⁷ For the solvent-graphene interaction, the surface energies of the solvent match that of graphene, so that it could balance the energy to exfoliate graphene.⁴⁸ Lange et al demonstrated that the scale-up of exfoliation could be achieved by shear exfoliation, ball milling or microfluidization.⁴⁹

Compared to the above, the oxidation–reduction of graphite is a more practical process for bulk-scale graphene materials. GO is generally fabricated by oxidizing graphite with a strong acid, mainly including Brodie, Staudenmaier, and Hummers method.⁵⁰ Hummers is the most commonly

used one at present because it is relatively time-efficient and safe.⁵¹ However, a conspicuous disadvantage of oxidation–reduction methods is that the products are generally multi-layer graphene or graphite microcrystals with different numbers of sheets, instead of monolayer nanosheets. Chemical vapor deposition is typically employed to fabricate monolayer graphene films on transition metals.^{52,53} Hydrocarbon gas (such as methane, ethane, or propane) is introduced into the reaction chamber as a carbon source. Graphene could be synthesized in this method with very few metallic residuals.⁵⁴ Tracy et al reported a novel method for low-temperature synthesis of monolayer graphene at 450°C on a polycrystalline bimetal Ni-Au catalyst. The electron beam co-deposition of the bimetal catalyst is the key procedure that enables the elimination of the pre-growth high-temperature annealing of the catalyst.⁵⁵

Biological Properties of GBNs

The unique biological properties of GBNs determine their wide application in the health care industry. Here, we mainly emphasize the properties of graphene-based materials relevant to tissue engineering. The most prominent feature of graphene-based materials is their high specific

surface area; this enables them to be loaded or to interact with not only inorganic molecules but also bioactive cells and vesicles. The morphology of GBNs could be observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM), manifested as randomly aggregated, flake-like sheets with folds on the porous surface, with or without face-to-face stacking of sheets⁴³ (Figure 2). Lee et al measured the mechanical strength of monolayer graphene membranes by atomic force microscope (AFM) nanoindentation.⁵⁶ In graphene-based composites, the mechanical nature of other soft materials can be significantly improved by graphene.^{57,58} The oxidation process damages the structure and mechanical properties of GO and other derivatives to some extent, nevertheless, both of them also appeal to researchers.⁵⁹ The GO allowed the sustained release of N-acetyl cysteine (NAC), known as a clinically applied antioxidant to reduce the reactive oxygen species (ROS). The hybrid membrane incorporating NAC, collagen I, and GO exhibited more excellent mechanical properties and water retention capacity compared to collagen-only scaffold.⁶⁰ Likewise, GO improves the mechanical properties of collagen membranes, as well as the roughness of the membrane surface slightly. These

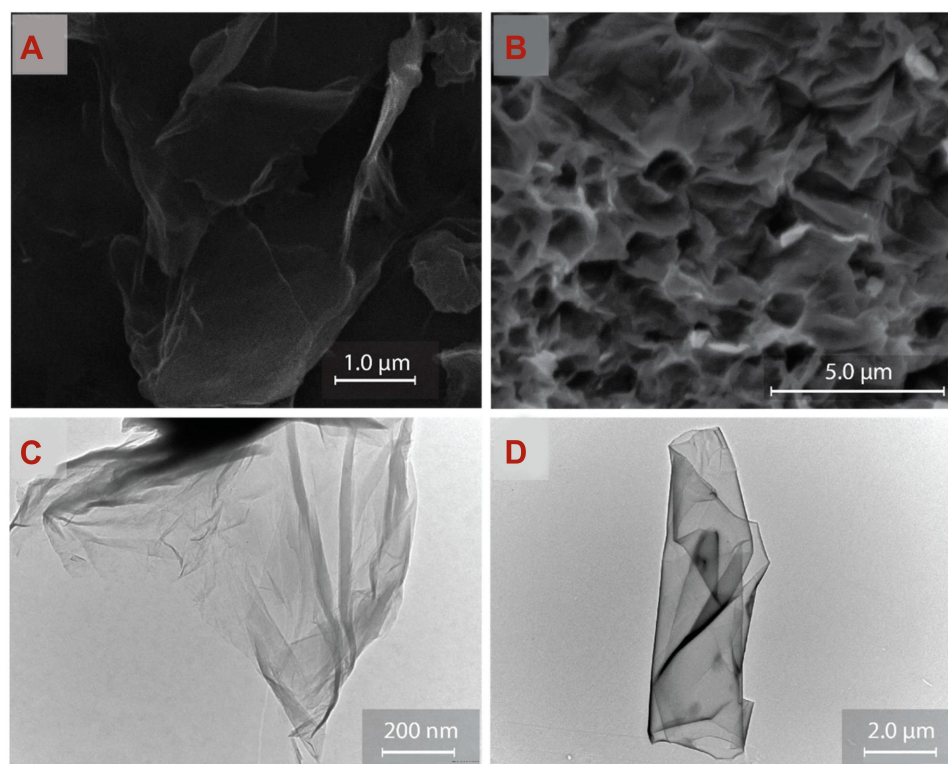


Figure 2 SEM morphology of (A) GO and (B) rGO. TEM images of (C) GO and (D) rGO.

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changes in performance have been detected as beneficial to cell proliferation and adhesion.⁶¹ Aerogels made of natural polymer materials have been widely employed in artificial bone transplantation because of their high porosity and great biocompatibility. But due to their poor mechanical properties, natural polymer aerogels appeared to be unqualified for large bone defects repair. Compositing of GO for preparation of Col I aerogels enhanced the compressive modulus dose-dependently, so that more appropriate mechanical properties were achieved.⁶²

The hydrophobic and electrostatic interaction could potentially increase the binding of graphene to other substances and improve several biological effects. The alkyl chains of graphene induce a high antiviral activity by secondary hydrophobic interactions. A perfect synergy between electrostatic and hydrophobic interactions induces a higher antiviral activity of graphene sheets.⁶³ Similarly, by fine-tuning of molecular interaction between peptide nanofibers and graphene, hybrid hydrogels with tailored properties can be designed.⁶⁴ However, even though the hydrophobic nature of graphene contributes to absorbing various hydrophobic organic molecules or polymers via van der Waals interaction, in terms of graphene, due to the hydrophobic nature, there arises a serious problem of agglomerations formation. In the last few years, studied revealed that GO may be an appropriate alternative for unique physicochemical properties, such as wide surface area, hydrophilia, flexibility, excellent electrical and thermal conductivity, and biocompatibility.⁶⁵ In particular, oxygen-containing functional groups on the surface result in hydrophilic nature, allowing dispersibility,⁶⁶ functional chemical modification and loading therapeutic components through both noncovalent interactions and covalent bonds via chemical reactions.

The excellent properties of GBNs for pathogens binding and inhibition have recently attracted much attention. The electrostatic interaction is the main driving force for binding graphene sheets to herpes simplex virus type 1 (HSV-1),⁶³ while the antibacterial activity of graphene sheets mainly depends on the collision between sharp edges and bacteria, that is, “nano-knives” mechanism.⁶⁷ Kim et al⁶⁸ have prepared an effective antibacterial surface consisting of GO and molybdenum disulfide (MoS). It exhibits a promising antimicrobial property toward the Gram-negative bacteria *Escherichia coli*. The graphene derivatives-containing scaffolds are also employed to kill the captured bacteria by IR-laser irradiation of bacteria-graphene complex due to thermal IR-absorption properties

of graphene,⁶⁹ and this is a typical example for GBNs as photothermal therapy reagent (Figure 3). In addition, GBNs display a prominent ability of heat conduction⁷⁰ and charge transport,⁷¹ inducing definite effects on target cells and tissues in biomedical application.

Biocompatibility of GBNs

Cytocompatibility and Cytotoxicity

The cytocompatibility of GBNs with several cell lineages has been confirmed in recent years. Pristine graphene is found to promote adhesion and proliferation of murine fibroblasts L929 cell line within 24 h of culture, and seemingly no cytotoxicity appeared. Migration of cells on the surface of graphene substrate acts better in comparison to the uncoated glass surface.⁷² By introducing hydrophilic groups (nitrogen ions) into 3D graphene, L929 cells are detected in better growth condition.⁷³ In bone tissue engineering, calcium silicate/graphene composites have been considered cell-friendly, and especially with a higher content of graphene were more favorable to the proliferation of human marrow mesenchymal stem cells.⁷⁴ For peptide hydrogels, the addition of GO and rGO has no impact on the overall cytotoxicity to 3D-cultured hMSCs at pH 6 over 14 days.⁶⁴ On a 3D printed nanocomposite hydrogel filled with GO, the interaction of GO with the polymer network by hydrogen bonds allows a slow release of GO to the culture medium. hADSCs adhered to the scaffolds survive and distribute orderly, regardless of the formulation of the ink.⁷⁵ In short, various factors like fabrication processes, dispersion in the medium, interacted functional groups all participate in the cells-GBNs performance.^{76,77}

However, the potential toxicity triggers a series of safety concerns in the biomedicine application of GBNs, which is essential for the clinical translation. Wang et al⁷⁸ found a dose-dependent relationship between GO and its cytotoxicity on human fibroblasts. When the GO concentration is less than 20 $\mu\text{g/mL}$, GO is nearly non-toxic to fibroblasts, but when the GO concentration is greater than 50 $\mu\text{g/mL}$, GO shows obvious cytotoxicity to fibroblasts. It mainly includes reducing cell adhesion and inducing apoptosis. It seems that GBNs induce a cytotoxic effect through a sustained mitochondrial depolarization. The increased ROS production mediates the depolarization, following the activation of NADH dehydrogenase and xanthine oxidase.⁷⁹ Analogously, though fluorinated graphene could improve cell adhesion on its surface in the early period, the intracellular ROS level is enhanced and the viability of MSCs declined after a longer incubation

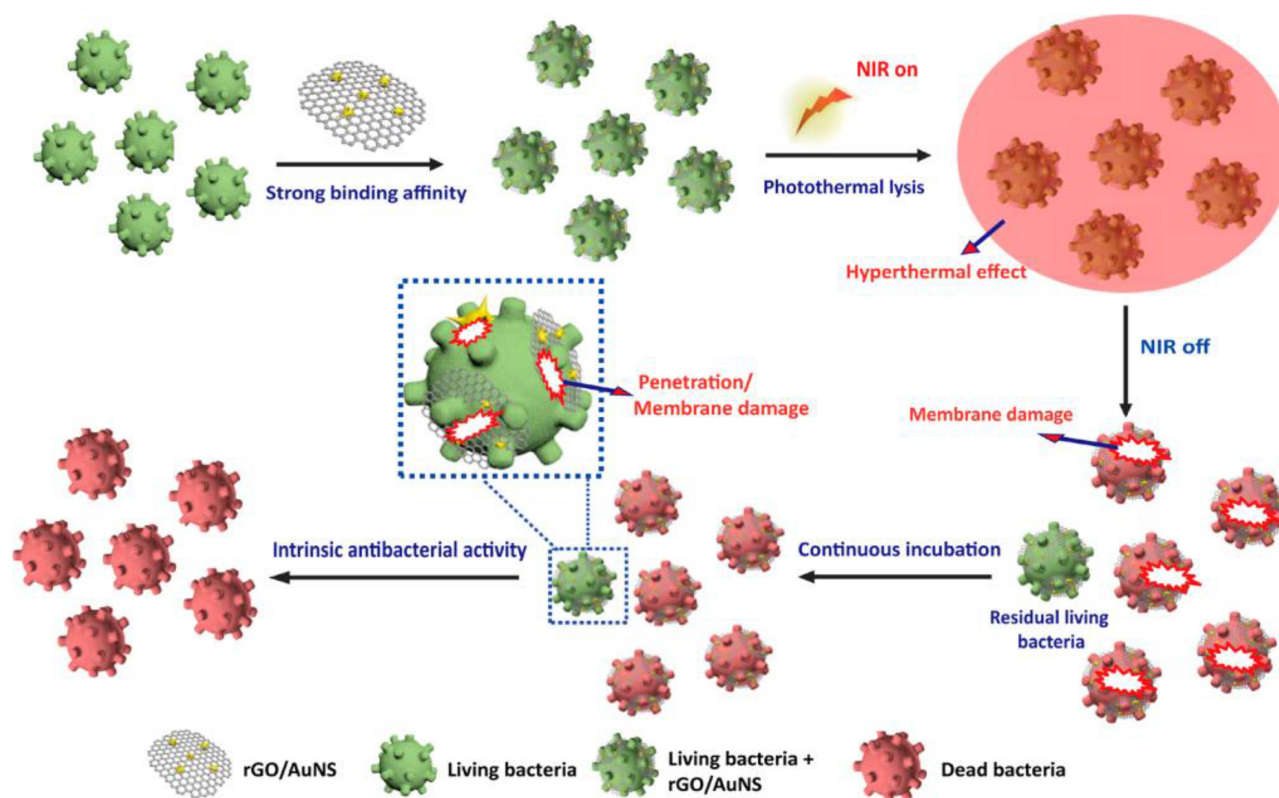


Figure 3 Schematic illustration of rGO/AuNS triggering intrinsic sterilization and antibacterial photothermal lysis.

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period.⁸⁰ Numerous functionalization methods are available to alleviate the cytotoxic response of graphene nanomaterials.⁸¹ GBNs coated with chitosan polymer exhibit reduced cytotoxicity compared to non-coated GBNs, which is reflected in fewer cell-cycle defects and higher cell viability.⁸²

Interactions in Biological Systems

Once introduced into the systemic circulation, GBNs interact with the cells and biomolecules of the circulatory system, so hemocompatibility is a crucial index to evaluate the in-vivo compatibility. Geng et al have found that the red blood cells inoculated on fluorinated graphene exhibit no obvious hemolysis phenomenon, and the morphologies of platelets reveal that the pseudopods outstretch well on the surface.⁸⁰ When the graphene quantum dots are injected (i.o) in mice, the uptake displays an age-related trend. It is, there is a decrease in the uptake of graphene quantum dots in aged mice when compared to young mice.⁸³ To assess the response of the human epidermis to graphene exposure more realistically, instead of interfered with protein corona

formation in the cell culture medium, a non-animal test has been implemented using the SkinEthic™ Reconstructed human Epidermis (RhE). The results confirm that the surfactants used to prepare GBNS, such as sodium dodecyl sulfate (SDS), are the initiators of the irritation, rather than the graphene material itself because the material is further washed by exfoliation is assessed non-irritant.⁸⁴

Duch et al⁸⁵ have found that the dispersion of GBNs and oxidation of the carbon backbone mattered in pulmonary toxicity. GO administered directly to the lungs of mice could cause severe and persistent lung injury with alveolar exudates and hyaline membrane formation. By contrast, inflammatory findings can be minimized for the intratracheally administration of highly dispersed nanoscale graphene. This difference is mainly due to the distinct levels of macrophage apoptosis. After intraperitoneal injection of GO nanoplatelets into adult male Wistar rats, a systematic toxicity appears.⁸⁶ Histopathological analysis reveals dose-dependent lesions in the liver, kidney, spleen, lung, intestine, and brain 21 days after GO nanoplatelets application. A low dose (50 or 150 mg/kg) of GO does not exhibit body weight

change, but a high dose (500 mg/kg) does. It has been demonstrated that the stable functionalization of GBNs could improve their in vivo pharmacokinetics and biodegradation, as well as minimize the potential toxicity.⁸⁷ For instance, by conjugating polyethylene glycol (PEG) to GO via a cleavable disulfide bond, a composite product with negligible toxicity and ideal degradability can be obtained.⁸⁸

Application and Putative Mechanisms in Cutaneous Repairment

Applications of GBNs as antibacterial agents have been reported,⁸⁹ and there are immense synergistic actions with empirical antibiotics. For instance, pristine GO could disrupt the bacterial membrane, convenient for the bound antibiotics to inactivate bacteria. Coupled with the minimal toxicity to keratinocytes when the GO concentration is <100 $\mu\text{g/mL}$, it suggests a broad prospect for application in infected wounds.⁹⁰ Ultrasonication could increase the dispersion and stability of the GO suspension, in the meanwhile, remove part of the oxygen-containing functional groups on the surface of nanosheets. In a rat excisional skin defect model, the wound healing rate of 1% UGO group is even about the same as the group of basic fibroblast growth factor (bFGF).⁹¹

In recent years, the ease of modification and functionalization, as well as fabrication of composites enable the utilization of biocompatible GBNs in cutaneous repairment.⁸¹ Tissue engineering utilizes different GBNs-incorporated composite scaffold materials to provide a suitable microenvironment for cellular behavior (Figures 4 and 5). To enhance the healing efficacy via promoting multiple wound healing stages in sequence, the ceria-graphene nanocomposites have been designed.⁹² In detail, the semiconductor ceria nanoparticles can inactivate bacteria by ROS generation under white light irradiation at the inflammation stage. When stepping into the proliferation stage, the ceria and graphene

nanoparticles would be separated. The former enter fibroblasts and scarily intracellular ROS, and the latter could act as a scaffold for fibroblasts migration. Thangavel et al⁹³ developed the rGO loaded isabgol nanocomposite scaffolds (Isab +rGO) to improve wound healing in normal and diabetic rats. 2, 2-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay revealed a prominent feature of antioxidation, which could minimize the ROS generation and lipid peroxidation. The natural polyphenolic compound curcumin has been reported for its wound healing properties including antioxidation, anti-inflammatory, reepithelization, and tissue remodeling. The scaffold incorporating rGO/Ag nanocomposites, curcumin or both of them has been prepared.⁹⁴ rGO/Ag nanocomposites increase the surface hydrophilicity of the scaffold, and therefore, the wettability is enhanced and cellular attachment on the scaffold is facilitated.

The capacity of absorbing excess exudates produced by the wounds is one of the typical advantages of hydrogels. Gelatin-methacryloyl (GelMA) based hydrogels can be swollen with around 1000% water during short-term immersion.⁹⁵ The prepared GelMA hydrogel loaded with rGO showed angiogenic potential in chicken embryo angiogenesis (CEO) testing. Liang et al⁹⁶ prepared a series of adhesive hydrogels for wound dressing based on hyaluronic acid (HA) -graft-dopamine (DA) and rGO using an H₂O₂/HPR (horseradish peroxidase) system. Owing to excellent electrical conductivity, rGO improves the wound-healing effects when incorporated into HA-DA hydrogel dressing. By immunohistochemical staining, on the 7th and 14th days, the level of CD31 in the wounds treated with HA-DA/rGO3 was detected higher than that in the Tegaderm membrane group, illustrating an improving angiogenesis process induced by the composite hydrogel. Furthermore, the GO can be reduced by polydopamine (PDA) and endows the chitosan/silk fibroin scaffolds with good electrical conductivity.⁹⁷ Generally, electroactive scaffolds as

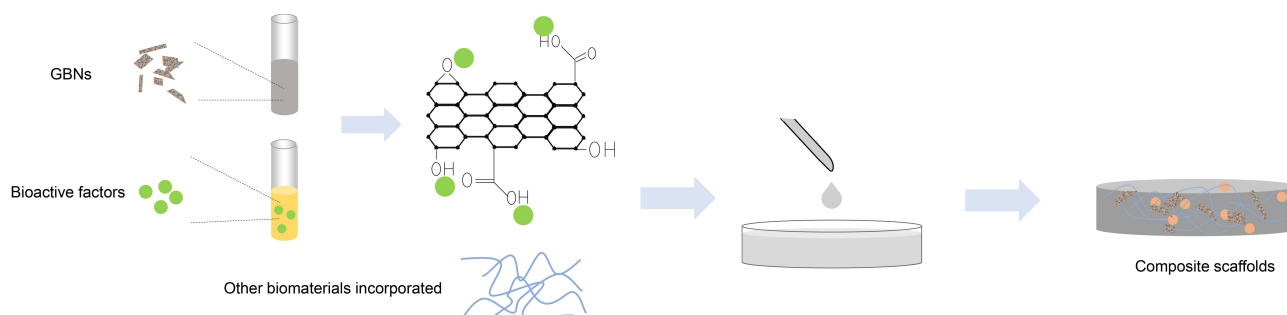


Figure 4 Schematic illustration of the common methods for preparing GBNs-incorporated composite scaffolds and wound dressings. GBNs could be combined with other biomaterials and drugs. In addition to enhancing the mechanical properties, GBNs could also improve the biological interactions by providing more anchoring sites for bioactive growth factors or specific drugs, utilizing the oxygen-containing functional groups of the basal plane and over the edges of the sheets.

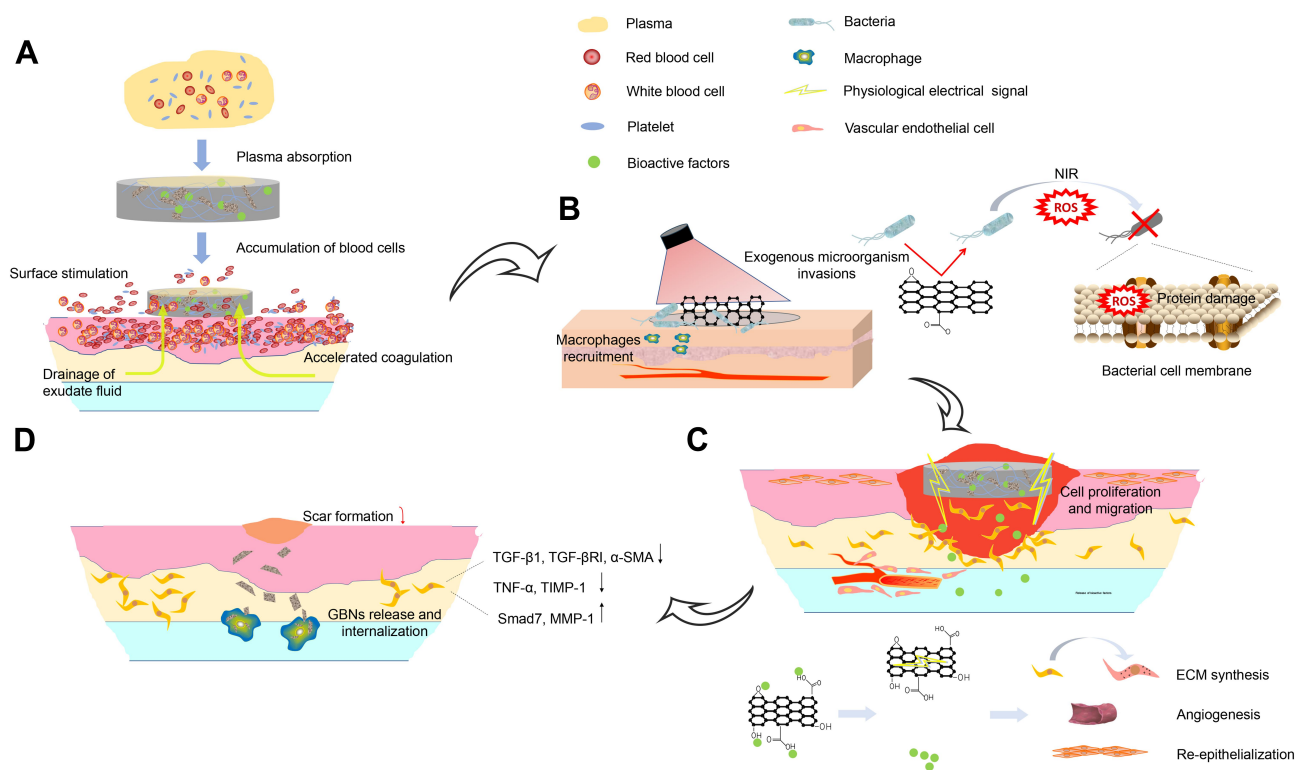


Figure 5 Schematic illustration of effect and putative mechanisms of GBNs on wound healing in multiple wound healing stages in sequence. **(A)** In the hemostasis phase, the high specific surface area enables nanofibers to absorb plasma fast. The oxygen polar groups can instantly stimulate erythrocytes and platelets at the interface, further promoting blood coagulation. **(B)** The composite scaffolds exhibit good antibacterial performance owing to the small pore size to inhibit microorganism invasions and the photothermal properties of GBNs. **(C)** Released bioactive factors and GBNs act synergistically on fibroblasts, keratinocytes and endotheliocytes et al during proliferation phase. **(D)** The presence of GBNs inhibits the fibroblasts from overactivation, accompanied by the down-regulation of scar-related genes. Highly expressed MMP in the extracellular matrix could facilitate the macrophage to internalize the GBNs through endocytosis, leading to the biodegradation.

described above could accelerate wound healing because they were able to respond to physiological electrical signals at wound sites during the healing process. Currently, most of the wound dressing hydrogels are chemically cross-linked, inducing a potential toxicity of residual organic cross-linkers. To avoid this, Yan et al⁹⁸ designed an injectable hydrogel formed in situ by physical crosslinking. This PEP- Ag@rGO hydrogel could be administered by spraying the hybrid aqueous mixture onto the targeted skin area and transit immediately to the hydrogel in response to a temperature higher than 30°C, thereby providing a stable dressing for the wound.

Aerogel is a gel material with gas as a dispersion medium. The solid phase of which is not only nano-scale but also porous. For instance, GO-COL aerogels⁶² have been observed a better repairing effect compared to aerogels prepared with Col only. Mellado et al⁹⁹ generated dry and stable composite aerogels based on GO and poly (vinyl alcohol) (PVA) combined with natural extracts through the sol-gel method. This aerogel promotes hemostasis via reduction of the coagulation time during contact with whole blood. GO was likely to promote fibroblasts migration and proliferation when

composited into collagen-N-acetyl cysteine hybrid membrane. In the rat cutaneous wound model, there appeared a full healing on the 14th day after the application of the hybrid membrane which preceded the control group markedly. Furthermore, the GO-incorporated hybrid membrane noticeably down-regulated the scar-related gene expression, which revealed a potential effect to facilitate scar-free wound closure.⁶⁰ By mimicking the hierarchical microstructure of nacre, GO-chitosan-calcium silicate film prepared by Xue et al¹⁰⁰ has a “brick-and-mortar” layered nanostructure and orderly porous lamellar micron-scale structure, endowing the film not only good tensile strength but also desirable breathability and water absorption property.

Application in Drugs Delivery and Cell Scaffolds

GBNs could load drugs noncovalently via π - π stacking interactions, hydrogen bonding, or hydrophobic interactions. The ability to cross cell membranes and the high specific surface area supply important advantages of

GBNs for drug delivery, and related studies have been widely conducted.⁵⁹ For instance, GO could efficiently deliver chlorogenic acid (CA) under phosphate buffer solution after forming a CAGO nanocomposite. Thermal analyses showed enhancement in the thermal stability of CAGO nanocomposite compared to the free drugs.¹⁰¹ The coating of GO on drug-delivery systems could also lead to a controlled release because of the intermolecular interactions (π - π stacking, hydrogen-bonding or electrostatic retardation) between the diffusing drugs and the decorations.¹⁰² They possess a greater advantage of delivering drugs at the targeted site with ease at a higher rate. Moreover, GBNs can easily be monitored and, even imaged *in vivo* by some functionalization methods, for example, incorporating with superparamagnetic nanoparticles as carriers of therapeutic factors.¹⁰³

GO exhibits a high drug loading capacity for aromatic molecules. It could release drugs and translocate into the nucleus slowly, which is pH-sensitive and initiated by NIR.¹⁰⁴ A promising drug delivery system ought to avoid initial burst release and loss of bioactivity of drugs in *in vitro* and *in vivo* applications.¹⁰⁵ The conjugation of PEG can weaken the bond between GBNs and drugs, which could induce a better drug release and treatment effect. Fourier transform-infrared spectroscopy (FT-IR) confirms that the π - π stacking interaction mediates the binding of drugs to the surface of GBNs.¹⁰⁶ Although the π - π interactions are important in drug loading, the dominant mechanism is the electrostatic interaction of ionized drugs with GO (especially through H-bonding). Rasoulzadeh et al tested the loading and releasing behavior of DOX, indicating that the nanocomposite hydrogels have better drug loading properties in comparison to pure hydrogel.¹⁰⁷ These nanocomposite materials usually exhibit excellent water dispersity and stability,¹⁰⁸ which provides prospects for clinical applications.

The various physiochemical properties of graphene nanomaterials permit a favorable microenvironment for the enhanced growth of cells or thus provide required stimuli for cellular differentiation to specific cell lineage. Wu et al¹⁰⁹ prepared starch nanofibers with incorporated GO by electrospinning. The viability of osteoblasts cell line MG 63 cultured on it remains unaltered in the case of appropriate GO concentration. GO promotes mineralization of calcium and phosphorus in a serum-like medium during the cell culture. The application of GO as a nano-filler into self-assembling peptide not only benefits for mimicking the mechanical properties of nucleus pulposus

but also promotes high viability and remains a metabolic activity of bovine nucleus pulposus cells over the 7 days of culture.¹¹⁰

GBNs also show the potential to fabricate alternative tools that can substantially affect gene expression of locally resident cells and change the tissue microenvironment.¹¹¹ Graphene could act as a biocompatible and conductive substrate for human induced pluripotent stem cells (hiPSCs) and mimic the biomimetic conductive cardiogenic niche to promote the self-renewal and cardiac differentiation of hiPSCs.^{112,113} It is reported that highly conductive GBNs, such as the carbon nanotubes, could enhance the neuronal differentiation of multipotent autologous cells in most cases. On the contrary, several GBNs-conjugated composite scaffolds which are less conductive appeared to boost the expression of myogenic-lineage marker genes.¹¹³ Saravanabhavan et al developed a GO functionalized chitosan nanoparticle to carry osteosarcoma cells-targeted siRNA. It shows a controlled pH-related release so that it facilitates the targeting of acidic tumor site^{114,115} (Table 1).

MSC and MSC-Exos MSCs in Cutaneous Wound Healing and Joint Application with GBNs

In the field of regenerative medicine, mesenchymal stem cells (MSCs) have been identified as a critical element for outstanding capacities including proliferation, multipotent differentiation, and bioactive paracrine factors release. MSCs derived from various tissues, such as bone marrow, subcutaneous adipose tissue and umbilical cord, are demonstrated a therapeutic potential for wound healing disorders. Previously, MSCs are usually implanted as a cell therapy by intravenous administration or subcutaneous injection around the wound.¹¹⁶ In fact, the healing effect is dependent on not only the localization of implanted MSCs but also their survival rate within the wound site and the induced microenvironment change. The features of high cell yields and being simply obtainable make MSCs advantageous to be utilized in tissue-engineering scaffolds. Based on materials science, many kinds of nanomaterials have emerged and been developed as biomimetic models to optimize the stem cell functions.⁸⁷

Due to the interactions between graphene and stem cells, extensive research has been devoted to developing GBNs that are capable of imitating the physiological

Table 1 A List of Different Types of GBNs Used in Biomedical Applications

GBNs	Type of Cells or Tissue	Effect	Ref.
CS/graphene composites	Human marrow stem cells	Cell-friendly, and higher contents of graphene were more favorable to the proliferation of cells.	[74]
GO and rGO hydrogel	hADSCs	hADSCs adhered on the scaffolds survived and distributed orderly.	[75]
GO suspension	Rat excisional skin defect	One of GO concentration group even exhibited about the same wound healing rate as the bFGF.	[91]
Ceria-graphene nanocomposites	Dermal fibroblasts	The ceria entered fibroblasts scarifying intracellular ROS, and graphene could act as a scaffold for fibroblasts migration.	[92]
PEGylated nano GO	HeLa cells	Release drugs and translocate into the nucleus slowly, and the nucleus translocation was NIR initiated and pH sensitive.	[104]
GO-incorporated starch nanofibers	Osteoblasts cell (MG 63)	GO promoted mineralization of calcium and phosphorus	[109]
GO- incorporated self-assembling peptide	Bovine nucleus pulposus cells	Cellular viability and metabolic activity were promoted over the 7 days of culture, which benefiting for mimicking the mechanical properties of nucleus pulposus,	[110]
Graphene	hiPSCs	Graphene mimicked the biomimetic conductive cardiogenic niche and promoted self-renewal and cardiac differentiation of hiPSCs.	[112]
PLLA and rGO, CNT, CNHs	hCMCs	Highly conductive CNTs boost neuronal differentiation, while less conductive CNH, RGO@PLLA, and PLLA scaffolds enhance the expression of myogenic markers.	[113]
PEG diamine/R8-functionalized GO	Breast cancer cell	The effective uptake of the nanocarrier by the cells shows superior cytocompatibility, and protects the siRNA and pDNA against enzyme degradation.	[115]
GO functionalized chitosan nanoparticle	Osteosarcoma cells (Saos-2 and MG-63)	The osteosarcoma cells-targeted siRNA loaded by composite nanoparticles were released in a controlled fashion at acidic pH	[114]

Abbreviations: CS, calcium silicate; hADSCs, human adipose-derived stem cells; bFGF, basic fibroblast growth factor; ROS, reactive oxygen species; NIR, near-infrared ray; PLLA, poly-L-lactic acid; CNH, carbon nanohorn; hCMCs, human circulating multipotent stem cells.

microenvironment of MSCs and controlling their fate.^{72,117–119} In diabetic wound repair, nanomaterial-drug-collagen hybrid scaffolds have been fabricated based on the PEGylated GO and acellular dermal matrix (ADM). Excellent cytocompatibility for GFP-labeled MSCs, as well as the induced adipogenic and osteogenic differentiation, is detected by two-photon excitation fluorescence and second-harmonic generation (TPEF-SHG) microscopy¹²⁰ (Figure 6). The combined application of growth factors or other active molecules also broadens the prospects of the GBNs with MSCs. Incorporation of GO into hybrid hydrogel prolonged the in vitro TGF- β 3 retention for up to 4 weeks.¹²¹ However, cell transplantation of MSCs is still associated with problems including safety concerns for potential tumorigenicity and immunological rejection. The exosomes derived from MSCs have the potential to avoid this and, meanwhile, maintain the paracrine secretome of the specific MSCs.

MSC-Exos: An Emerging Cell-Free Approach to Cutaneous Wound Repair

MSC-Exos are considered to be one of the most important secreted products of MSCs. Due to its high stability, non-immune rejection, and easy-to-control concentration, it could mediate intercellular communication and enhance wound healing.¹²²

Li et al¹²³ studied the effect of human umbilical cord mesenchymal stem cells (hUMSCs) on the inflammatory response in diabetic rat burn models. They have found that the endogenous miR-181c of hUMSC-Exos can inhibit Toll-like Receptor 4 signaling pathway, thereby attenuating the lipopolysaccharide-mediated inflammation. After administration of hUMSC-Exos overexpressing miR-181c, the expression of inflammatory factors such as TNF- α and IL-1 β is up-regulated, while the anti-inflammatory factor IL-10 is down-regulated. It is found that Exosomes derived from adipose-derived stem cells (ADSC-Exos) may be taken up

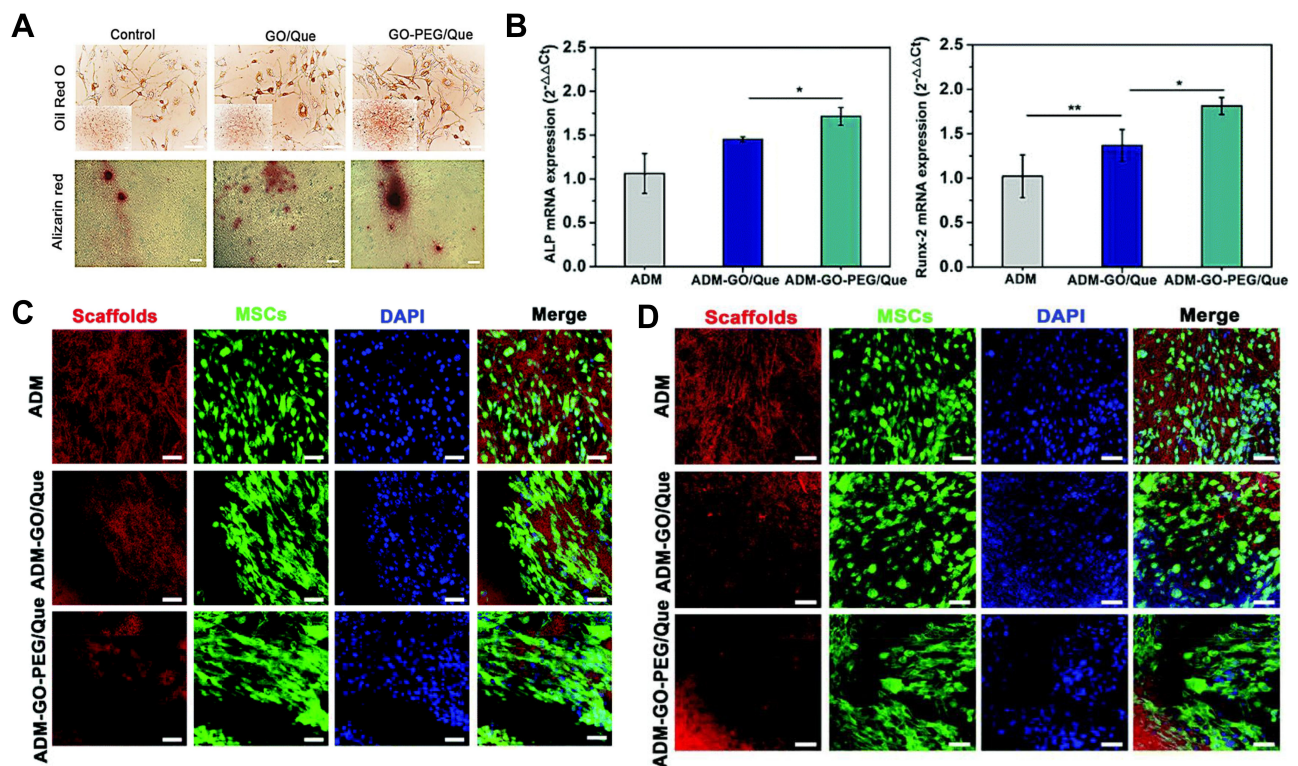


Figure 6 (A) Positive oil red O staining of MSCs after incubation with GO/Que or GO-PEG/Que indicates their potential to differentiate into adipocytes. **(B)** Quantitative analysis of the mRNA expression of ALP and Runx-2 of MSCs seeded on the ADM, ADM-GO/Que, and ADM-GO-PEG/Que scaffolds (* $p \leq 0.05$, ** $p \leq 0.01$, $n = 3$). TPEF-SHG imaging showed that the PEGylated GO exhibited improved **(C)** osteogenic and **(D)** adipogenic differentiation of MSCs on the scaffolds. Scale bar: 50 μm .

Notes: Reprinted from Jing Chu, Panpan Shi, Wenxia Yan et al. PEGylated graphene oxide-mediated quercetin-modified collagen hybrid scaffold for enhancement of MSCs differentiation potential and diabetic wound healing. *Nanoscale*. 2018;10(20):9547–9560. Copyright 2018, with permission from The Royal Society of Chemistry.¹²⁰

and internalized by dermal fibroblasts to stimulate migration, proliferation and collagen synthesis in a dose-dependent manner with increased expression of N-cadherin, cyclin-1, PCNA and collagen I, III¹²⁴ (Figure 7). Our group has demonstrated that the ADSC-Exos could act on the dermal fibroblasts in vitro and in vivo via PI3K/Akt signaling pathway, so that optimize the collagen deposition and wound closure.¹²⁵ Nonetheless, hUMSCs-Exos inhibits the proliferation and migration of fibroblasts to prevent excessive tissue proliferation under high cell density.¹²⁶ The hUMSCs-Exos-derived protein 14-3-3 ζ contributes to this effect by promoting the phosphorylation of protein YAP and activating the Hippo-YAP pathway, thereby having a reverse effect on the Wnt/ β -catenin signaling pathway.

Accessibility requirements for the source of exosome isolation spawn the application of mass-produced sample from the human body. The menstrual blood-derived mesenchymal stem cells (MenSC)-derived exosomes could resolve inflammation via induced M1-M2 macrophage polarization.¹²⁷ Exosomes derived from MSCs in human urine samples

(USC-Exos) have been demonstrated the ability of promoting angiogenesis of diabetic wounds, and its advantage lies in the simple, safe, non-invasive and low-cost isolation procedure.¹²⁸

Recent studies indicate that exosomes derived from modified or pretreated MSCs may exhibit a superior wound healing property. Ding et al¹²⁹ isolated exosomes from BMSCs preconditioned by deferoxamine. The obtained exosomes could activate pathways pivotal in skin wound healing, such as AKT, extracellular signaling kinase (ERK), and transcriptional activator 3 (STAT3). Blue light illumination of hUMSCs could enhance the proangiogenic ability of their exosomes in tissue repair with an increase in miR-135b-5p and miR-499a-3p expression.¹³⁰ Exosomes derived from MSCs transfected with lncRNA H19 could suppress apoptosis and inflammation of fibroblasts in diabetic foot ulcer (DFU). It has been observed that the lncRNA H19 in MSCs is transferred to fibroblasts through exosomes and functions through the lncRNA H19/miR-152-3p/PTEN axis.¹³¹

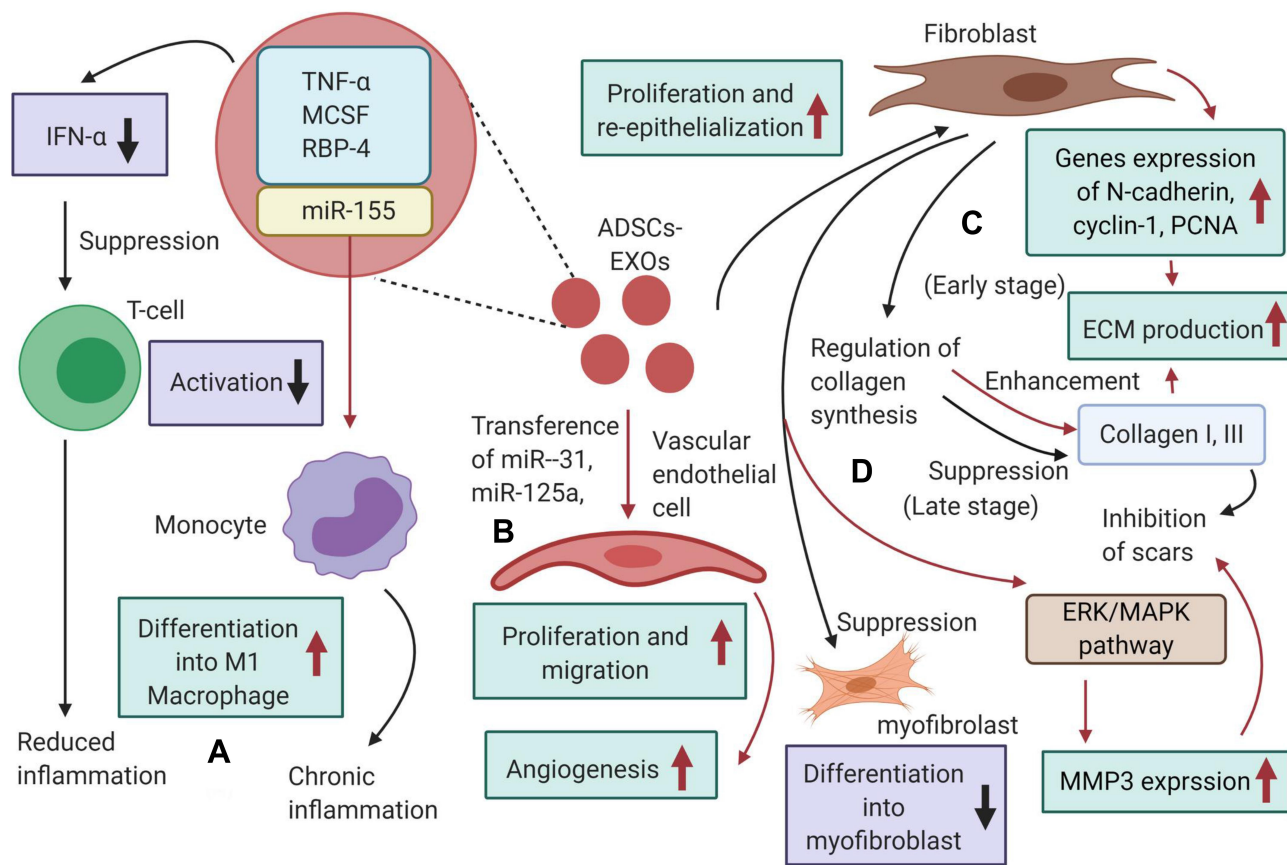


Figure 7 Mechanisms by which ADSCs-Exos could promote wound healing. **(A)** ADSCs-Exos contain immunoregulatory proteins and subsequently inhibit the activation of T cells, resulting in reduced inflammation. **(B)** ADSCs-Exos can transfer miRNA-125a and miRNA-31 to vascular endothelial cells, promoting angiogenesis. **(C)** In the proliferation phase, ADSCs-Exos could stimulate N-cadherin, cyclin-1, PCNA and collagen I, III expression and increase ECM production; **(D)** in the matrix remodeling phase, ADSCs-Exos prevent the differentiation of fibroblasts into myofibroblasts, and reduce scarring and activate the ERK/MAPK pathway to increase MMP3 expression. **Notes:** Reprinted from Yang An, Shuyan Lin, Xiaojie Tan et al. Exosomes from adipose-derived stem cells and application to skin wound healing. *Cell proliferation*. 2021; e12993. Copyright 2021, with permission from John Wiley & Sons Ltd.¹⁵¹

Exploration of Novel Effective MSC-Exos Administration Routes for Wound Repair

In most of the above studies, exosomes are applied through subcutaneous injection to several sites around the wound. Together with other common methods of administration, like intravenous injection, they face the challenges of burst release and rapid clearance. Therefore, it is not conducive to play the due role during several complex stages in the whole wound repair process. At present, researchers are moving towards a new strategy based on loading MSC-Exos by patches, injectable microcarriers or hydrogels, aimed at maintaining the function of exosomes at the wound site and enhancing efficiency and safety.

As for carrier materials, the controlled release function is required. Chitosan and relevant compounds are ideal carriers for the sustained release of nanoparticles including exosomes.^{82,132,133} The delivery of exosomes derived from

miR-126-3p-overexpressing synovium mesenchymal stem cells is markedly prolonged to achieve a long-term exosome exposure to the wound site. With the increase of immersion time in the conditioned medium, the number of labeled exosomes in the perinuclear region of microvascular endothelial cells slowly increases.¹³⁴ Shi et al prepared the chitosan/silk hydrogel sponge by freeze-drying method to be a scaffold for exosomes. Since chitosan is a hydrophilic polymer, this hydrogel sponge shows good swelling behavior, creates a moist environment and enhances the angiogenesis and neuronal ingrowth.¹³⁵ Alginate-based hydrogels have been designed to encapsulate ADSC-Exos to fabricate a bioactive scaffold,¹³⁶ which is tested biodegradable and biocompatible, reflecting potential as a cell-free therapy.

Several studies have shown that various artificial injectable hydrogels can not only promote the sustained

release but also increase the local retention of exosomes in vivo.^{135–137} An injectable, self-healing and antibacterial polypeptide-based FHE hydrogel (F127/OHA-EPL) has been developed with a stimuli-responsive exosomes release for enhancing chronic wound healing and complete skin regeneration.¹³⁸ The ureido-pyrimidinone supramolecular hydrogel undergoes a solution-to-gel transition when the pH is switched from high to neutral, with a threshold at $\text{pH} \approx 8.5$. Moreover, nanoparticle tracking analysis (NTA) shows that the size distribution profile remains normal despite encapsulated and released from the hydrogel.¹³⁷ Adipose tissue is abundant around the local cutaneous wound; thus, ADSC-Exos with bioactive constituents have earned boomed studies on exploring novel delivery systems for them. Likewise, adipose stromal cell-derived exosomes were released in a pH-dependent manner from injectable adhesive thermosensitive multifunctional dressing to promote angiogenesis in the diabetic wound.¹³⁹ An acellular tissue patch prepared from photoinduced imine crosslinking hydrogel glue, combined with stem cell-derived exosomes, can be well integrated with the cartilage matrix and promote cell deposition at cartilage defect. The unique delivery by patch matches the intricate

cartilage surface well, thus facilitating the contact of MSC-Exos with articular cartilage¹⁴⁰ (Figure 8).

In general, the exosome-carrier compound displays better healing outcomes than the exosomes or carrier materials alone, suggesting a synergistic effect through the sustained release of MSC-Exos. Further exploration of the putative mechanism is required which is essential to realize the transformation of research results into reliable clinical applications.

Outlook: Combined Application and Feasibility

In recent years, the cutaneous wound repair, as a complex process involving different types of resident and recruited circulating cells, has been well studied. The single-cell technologies allow unraveling the heterogeneous of these cell subsets as well as identifying and characterizing novel rare cell subsets, instead of detecting the average cellular output of biology states.¹⁴¹ It is revolutionary in understanding the mechanisms of normal and impaired wound repair.

Various natural¹⁴² and synthetic¹⁴³ materials loaded with Exos have been reported as cell-free therapeutic

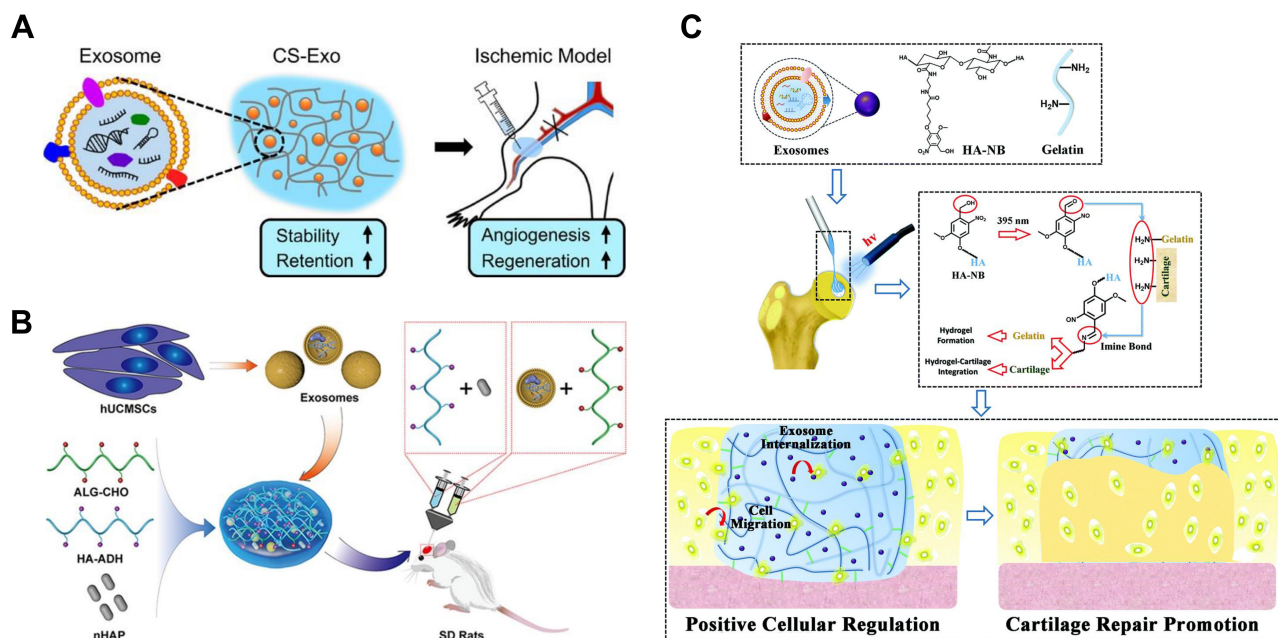


Figure 8 Typical schematic representations of MSC-Exos-incorporated hydrogels or scaffolds based on (A) chitosan, (B) sodium alginate, (C) hyaluronic acids for tissue regeneration.

Notes: (A) Reprinted from Kaiyue Zhang, Xiangnan Zhao, Xiaoniao Chen et al. Enhanced Therapeutic Effects of Mesenchymal Stem Cell-Derived Exosomes with an Injectable Hydrogel for Hindlimb Ischemia Treatment. *ACS Appl Mater Interfaces*. 2018;10(36):30081–30091. Copyright 2018, with permission from ACS publication.¹³² (B) Reprinted from Shuo Yang, Biao Zhu, Peng Yin et al. Integration of Human Umbilical Cord Mesenchymal Stem Cells-Derived Exosomes with Hydroxyapatite-Embedded Hyaluronic Acid-Alginate Hydrogel for Bone Regeneration. *ACS Biomater. Sci Eng*. 2020;6(3):1590–1602. Copyright 2020, with permission from ACS publication.¹⁵² (C) Reprinted from Xiaolin Liu, Yunlong Yang, Yan Li et al. Integration of stem cell-derived exosomes with in situ hydrogel glue as a promising tissue patch for articular cartilage regeneration. *Nanoscale*. 2017;9(13):4430–4438. Copyright 2017, with permission from The Royal Society of Chemistry.¹⁴⁰

applications for wound healing. This review proposes the joint application of GBNs and MSC-Exos, and strives to develop the GBNs as the delivery system of MSC-Exos. It matters whether the two can complement each other to achieve a good synergistic effect in wound repair. It is exciting to note that the high specific surface area and loading rate of GBNs would be helpful to increase the effective dose of MSC-Exos acting in local wound sites, as well as the strong sustained release ability allows MSC-Exos to obtain an enhanced lasting acting time, retention rate and stability. The interaction of GBNs with cells involved in wound healing response helps to increase the specificity of MSC-Exos to regulate gene expression. Coupled with the inherent antibacterial properties of GBNs, they get prospects to further improve the re-epithelialization, angiogenesis and collagen maturation, as for exploiting novel skin substitutes and cell-free therapies.

Additionally, the consociation of GBNs with 3D-bioprinting is also worthy of attention, to obtain tailor-made structures and functions for tissue engineering and 3D cell culture.^{144,145} Studies have revealed that the mixture of graphene into composite bio-inks helps to enhance the mechanical properties and printability.¹⁴⁶ The aforementioned biological properties of GBNs provide additional advantages in cell-containing bio-ink such as increasing cellular vitality in the matrix. During in vitro or in vivo culture of printed products, the anti-oxidant activity could play a role in alleviating ROS generation and lipid peroxidation.⁹⁵ The hydrophobic, hydrophilic, and π - π interactions endow GBNs with the ability to adsorb proteins from biological surrounding to the 3D construct.⁷⁵ The addition of GO could increase the cellular proliferation directionality along the printed threads, which offers a novel solution for the reconstruction of anisotropic structures, such as tendons and muscle fibers.

The bottlenecks faced include that the microcosmic mode and characteristics of contact between GBNs and MSC-Exo need to be explored in depth, so that to improve the attachment, release properties and compatibility with membrane structure. The balance between regenerative value and potent toxicity should be properly handled.¹⁴⁷ Furthermore, unlike extensively studied tumor cells, the yield of MSC-Exosomes is a limiting factor for large-scale production for cell-free therapies.¹⁴⁸ Therefore, it is of great necessity to increase the yield without reducing their therapeutic efficacy.¹⁴⁹

Disclosure

The authors reported no conflicts of interest for this work.

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