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Carbon nanomaterials and their interactions with bacteria

Uhlíkové nanomateriály a jejich interakce s bakteriemi

Bachelor's thesis

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Prohlášení:

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Signature:

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Abbreviations

AFM	atomic force microscopy
AOT	sodium bis(2-ethylhexyl) sulphosuccinate
CNT	carbon nanotube
CTAB	cetyltrimethylammonium bromide
DLC	diamond-like carbon
GSH	glutathion
HRTEM	high-resolution transmission microscopy
IGEPAL	tert-octylphenoxy poly(oxyethylene)ethanol
LDS	lithium dodecyl sulphate
MCD	microcrystalline diamond
MIC	minimal inhibition concentration
MWCNT	multi-walled carbon nanotube
nC_{60}	nano-fullerene C_{60}
NCD	nanocrystalline diamond
PVA	polyvinyl alcohol
PVP	polyvinylpyrollidone
SC	sodium cholate
SDBS	sodium dodecylbenzene sulphonate
SDS	sodium dodecyl sulphate
SEM	scanning electron microscopy
SWCNT	single-walled carbon nanotube
THF	tetrahydrofurane
TTAB	tetradecyl trimethyl ammonium bromide
UNCD	ultrananocrystalline diamond
ζ-potential	zeta-potential

Abstrakt

Uhlíkové nanomateriály jsou v poslední době v centru pozornosti hlavně díky svým zajímavým, často unikátním vlastnostem. Mají široké možnosti využití, například v elektronice, optice, kosmetice solárních článcích, stavebních materiálech, vzduchových filtrech, k leštění materiálů, jako ochranné povrchy či suchá maziva. Zatímco jejich fyzikální a chemické vlastnosti jsou již dobře prozkoumány, výzkum jejich působní na živé organismy je stále v počátcích. Tato práce je zaměřena na interakce uhlíkových nanomateriálů, konkrétně grafenu, fullerenu, uhlíkových nanotrubiček a nanodiamantů, s bakterálními buňkami a jejich antibakteriální a antiadhezivní účinky. Mechanismy toxického působení zahrnují porušení vnějších struktur buňky v důsledku přímého kontaktu s nanomateriálem, narušení bakteriálního metabolismu nebo produkci volných kyslíkových radikálů. Přesné porozumění dějům, které se odehrávají mezi bakteriální buňkou a uhlíkovými nanomateriály, může přispět k výzkumu jejich možných aplikací v medicíně či možností jejich ekologické recyklace.

Klíčová slova:

Nanomateriály, grafen, fulleren, uhlíkové nanotrubičky, nanodiamanty, antibakteriální účinky, antiadhesní účinky

Abstract

Recently, carbon nanomaterials gain attention especially for their interesting, often unique, properties. They can be used in wide range of applications, such as electronics, optics, cosmetics, solar cells, construction materials, air filters, polishing materials, protective coatings and dry lubricants. Whereas their physical and chemical attributes have already been intensively examined, the research on their effects on living organisms is still at the preliminary stage. This work is focused on the interactions of carbon nanomaterials, namely graphene, fullerene, carbon nanotubes and nanodiamonds, with bacterial cells and their antibacterial and antiadhesive properties. The mechanisms of the toxic action of carbon nanomaterials against bacteria include damage of outer cell structures as a consequence of the direct contact with a nanomaterial, impairment of bacterial metabolism or reactive oxygen species production. Exact understanding of the processes that take place between bacterial cell and carbon nanomaterials can contribute to the research on their medical applications and ecological recycling in the future.

Key words:

Nanomaterials, graphene, fullerene, carbon nanotubes, nanodiamonds, antibacterial properties, antiadhesive properties

1. Introduction

Nanomaterial is defined as a material whose size does not exceed 100 nm in at least one dimension. The size of nanomaterial is so small that quantum effects cannot be neglected and manifest in the properties of the material. Nanomaterials, which have been intensively studied in the last few decades, comprise many new materials with unique properties which may be of use in various sectors of industry. In connection with their ever-rising usage in many applications, including their potential future employment in medicine, new, rapidly evolving scientific disciplines, such as nanomedicine or nanotoxicology, are arising.

Carbon is the 17th most common element found on Earth and the most abundant element in living organisms. In nature, carbon can be found in amorphous form or in one of its crystalline forms, which comprise graphite and diamond. These forms substantially differ in their properties (see further). With development of nanotechnologies, new carbon nanomaterials, such as graphene, fullerene, carbon nanotubes or nanodiamonds, have been discovered and investigated. Due to their variability and superior properties, such as excellent conductivity, hardness or exquisite optical properties, they are considered promising materials for broad field of industrial applications, such as electronics, optics, sensors, probes, catalysts, high-strength materials and also for medical applications, such as articular implant coatings, drug delivery, substrates for tissue engineering and bio-labelling. Recently, their biocompatibility and possible harmful impacts on living organisms have also been intensively studied, as - with the prospect of their increasing usage - the release of nanomaterials to the environment is highly probable. Therefore, the investigation on their behaviour in natural environment and on possible methods of their ecological disposal or recycling is of high importance.

The research has recently been focused in majority on the interactions of nanomaterials with eukaryotic, especially human, cells and less is known about their impact on prokaryotes. Therefore, the aim of this thesis is to summarize the available information and record the current state of knowledge on this very interesting topic. I will also mention some practical information for working with these materials in a laboratory. Based on the information available in literature, I will also try to suggest some hypothesis for possible mechanism of action against microorganisms.

I will focus especially on the interactions of carbon nanomaterials, namely graphene, fullerene, carbon nanotubes and nanodiamonds, with bacteria, because I would like to follow this topic also in my future research.

2. Carbon nanomaterials

Carbon can be found in nature in the form of amorphous carbon or two crystalline allotropes - graphite and diamond. Carbon atoms can occur in three possible hybridization states: sp, sp^2 and sp^3. Hybridization is the linear combination of two or more electron orbitals and determines the number and direction of possible bonds in the molecule.

Carbon atoms in graphite and diamond differ in their hybridization and hence these two materials crystallize in different crystal order. Graphite atoms due to their sp^2 hybridization can make three planar bonds that are oriented at the angle of 120°. The structure of graphite can be seen in Figure 1 - every carbon atom can create three covalent bonds with neighbouring atoms which results in formation of planar sheets (called graphene – chapter 2.1). The bonds are quite strong, however the sheets are interconnected together only by noncovalent weak Van der Waals forces and so graphite is a very soft material (Lifshitz, 1999). In this work, I focus on three graphitic nanomaterials: graphene, fullerene and carbon nanotubes, properties and structure of which are described in chapter 2.1.

In contrast, carbon atoms in diamond are sp^3 hybridized and hence can make four bonds pointing to the vertexes of regular tetrahedron. Diamonds crystalize in facial centred cube crystal order and the structure is referred to as "diamond structure". There exist two types of diamonds according to their structure: cubic diamond (usually called just "diamond") and hexagonal diamond (also called "lonsdaleite"). Crystal order of both are very similar, the difference lies in the arrangement of the atoms, as can be seen from Figure 1. Most of the nanodiamonds (chapter 2.1) used in biological experiments are cubic type of diamonds. All the bonds in diamond are very strong which results in high hardness of the bulk diamond material.

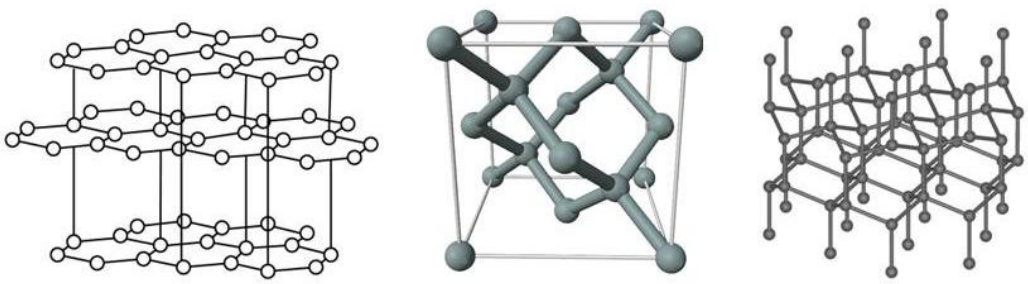


Figure 1 The structure of (**A**) graphite, (**B**) diamond, (**C**) lonsdaleite
Source: http://scmhardsoft.altervista.org/tag/curiosity
http://thefutureofthings.com/news/6677/harder-than-diamond.html

2.1. Structure, size and properties of carbon nanomaterials

Graphene can be described as a single layer of graphite. It is planar one-atom-thick sheet which forms 2D hexagonal lattice as shown in the Figure 2. It is believed that graphene is the thinnest material ever measured (Meyer *et al.*, 2007). In contrast to graphite, graphene is very resistant and tough. This is due to absence of weak Van der Waals forces which tie individual graphene sheets to each other in the structure of graphite. As mentioned above, the bonds between carbon atoms are very strong which results in high graphene durability and flexibility. Besides, graphene is also very conductive material. This is caused by remarkably high electron mobility due to its largely dislocated π-electrons (Brownson and Banks, 2010; Yang *et al.*, 2013).

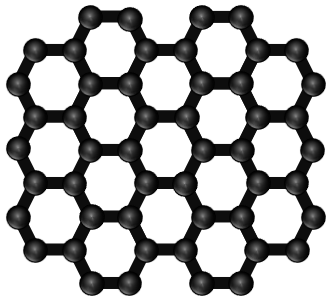


Figure 2 The structure of a graphene sheet

Carbon nanotubes (CNTs) are structurally more complicated than graphene and morfologically very interesting. A carbon nanotube can be described as one (or more) graphene sheet(s) rolled into the shape of a hollow tube, see Figure 3.

According to the number of graphene sheets rolled into a tube, we can distinguish between single-walled carbon nanotubes (SWCNTs, also called graphene nanotubes) and multi-walled carbon nanotubes (MWCNTs, also called graphite nanotubes or multi shell nanotubes).

SWCNTs are usually closed at the ends with a cap-like structure which arises during the process of

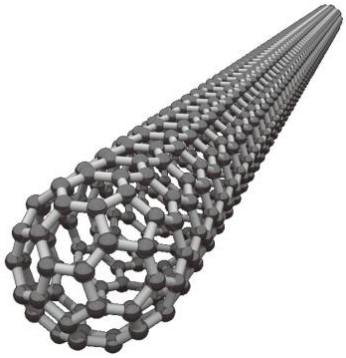


Figure 3 A model of a single-walled carbon nanotube (SWCNT) capped with a fullerene hemisphere (Uo *et al.*, 2011).

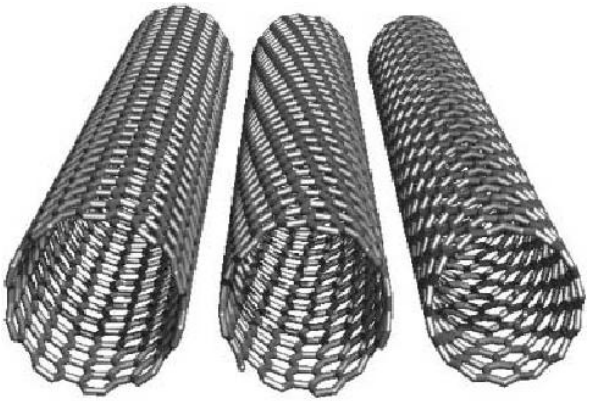


Figure 4 The chirality of single-walled carbon nanotubes (SWCNTs): **(A**) armchair, (**B**) chiral, (**C**) zigzag (Hirsch, 2002).

synthesis (Foldvari and Bagonluri, 2008). The smallest SWCNTs have been reported to have diameter about 0,4 nm (Wang *et al.*, 2001). Three possible structural configurations of carbon atoms exist in SWCNTs which are referred to as armchair, zigzag and chiral (or helical) arrangement, see Figure 4.

MWCNTs consist of more layers of graphene and therefore have external diameter above 10 nm (Beg *et al.*, 2011). According to their structure, MWCNTs can be divided in two groups. In so called "Russian doll" type MWCNTs, the graphene sheets are organized in concentric layers inserted into each other. In contrast, MWCNTs organized according to so called "parchment model" are formed by just one sheet of graphene rolled around itself (Beg *et al.*, 2011), see Figure 5. Some authors, such as Lavin (2002) suppose that these two forms can exist side-by-side in the same sample and even in one individual nanotube. In such case, both structures are separated from each other by well visible structural defects.

Similar to graphene, CNTs also are very stiff, tough and resistant. The yarns made from MWCNTs (Figure 6) possess great strength, toughness an resistance to knot-induced failure which predetermines them for usage in many applications (Zhang *et al.*, 2004). Other very interesting

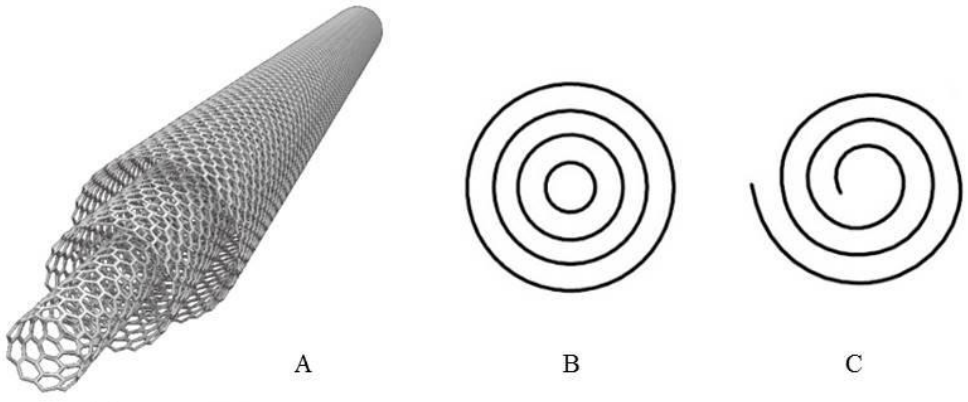


Figure 5 (A) A model of a "Russian doll" type multi-walled carbon nanotube (MWCNT) (**B**) schematic draft of the layer arrangement in a "Russian doll" and (**C**) "parchment" models of MWCNT
Modified from: (**A**) (Uo *et al.*, 2011); (**B**) and **C**) (Eletskii, 2004)

property of CNTs is their conductivity: CNTs can be conductive or semiconductive in dependence on their chirality: for example armchair nanotubes are always conductive while zigzag nanotubes are always semiconductive (Dresselhaus *et al.*, 2004). Detailed description of this phenomenon is described elsewhere (Joselevich, 2004).

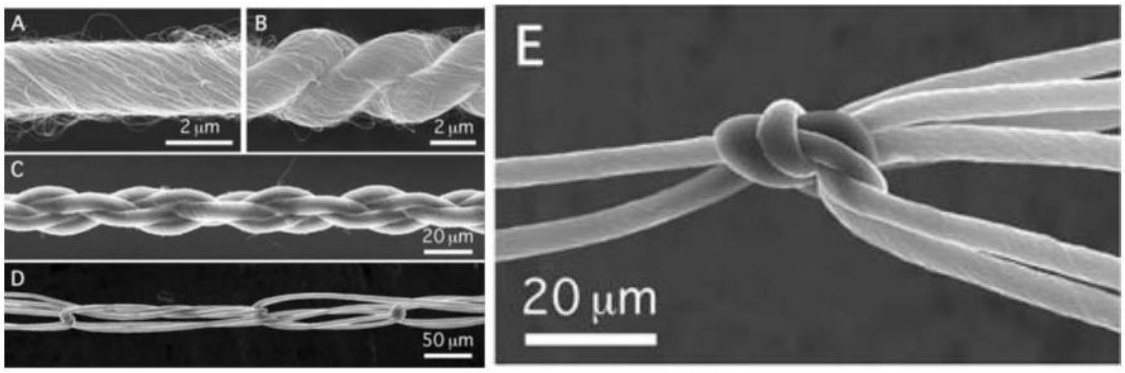


Figure 6 Scanning electron microscope (SEM) images of yarns made from multi-walled carbon nanotubes (MWCNTs): (**A**) single-ply, (**B**) two-ply, and (**C**) four-ply MWCNT yarns (**D**) knitted and (**E**) knotted MWCNT yarns (Zhang *et al.*, 2004).

The last graphite-derived nanomaterials are fullerenes. Spherical hollow shape is characteristic of these molecules. The most stable fullerene is fullerene C_{60} (also called buckminsterfullerene or buckyball) which consists of 60 carbon atoms. Its shape is a perfect sphere and resembles a football, see Figure 7A. The single molecule has diameter of approximately 0,7 nm (Kroto *et al.*, 1985).

There exist also fullerenes consisting of more or less atoms than 60, for example C_{28}, C_{32}, C_{50}, C_{70}, C_{63}, C_{83} etc. (Tsao *et al.*, 2002; Laowachirasuwan, 2008) whose shapes are not perfectly spherical as that of C_{60}. Fullerene molecules can be also formed by more than only one layer of carbon atoms – such multi-walled fullerenes are called nano-onions, see Figure 7B.

It should be emphasized, that in contrast to the terms "graphene" and "nanotube", which refer to materials, the term "fullerene" refers to an individual molecule. The fullerene nanoparticles are

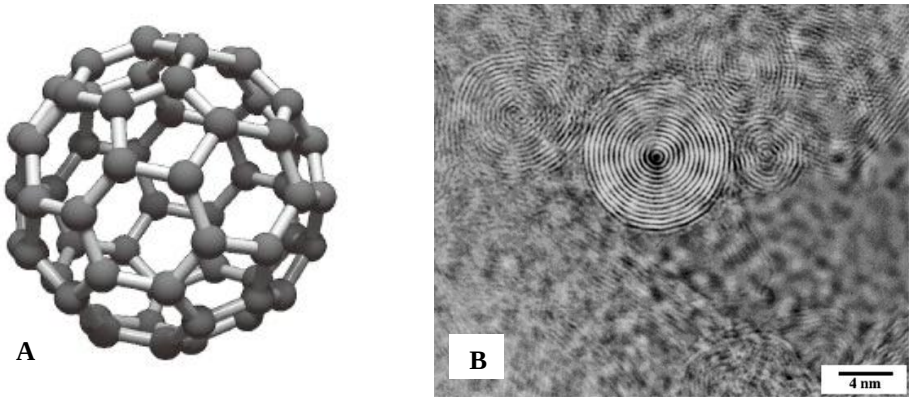
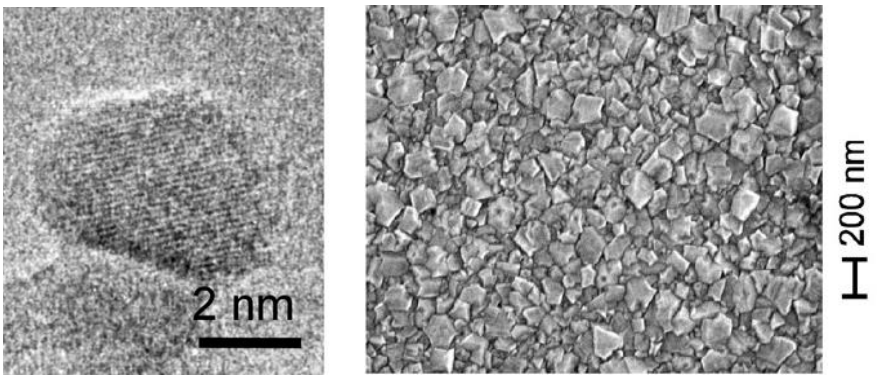


Figure 7 (A) The structure of the fullerene C_{60} molecule (Uo *et al.*, 2011) (**B**) High resolution transmission electron microscopy (HRTEM) image of a nano-onion. Concentric circles represent individual fullerene layers. Modified from (Roy *et al.*, 2003).

called nano-fullerenes and denoted nC_{60}, nC_{70} etc. Fullerene crystallizes, similar to diamond, in facial centred cube crystal order and its crystals are very hard and durable.

The structure of diamonds is totally different compared to the materials mentioned so far. Bulk diamonds crystallize in diamond cubic crystal structure and possess sp^3 hybridization (in contrast to graphite and other already mentioned carbon nanomaterials, which have sp^2 hybridization). Based on the size and crystallinity of diamond particles, we can distinguish between monocrystalline, polycrystalline, microcrystalline (MCD), nanocrystalline (NCD) and ultrananocrystalline (UNCD) diamonds (Sharda *et al.*, 2001). Monocrystalline diamonds are very expensive. These are the diamonds mainly used in jewellery. Polycrystalline, microcrystalline, nanocrystalline and ultrananocrystalline diamonds are cheaper and their production is easier than that of monocrystalline. The designation micro-, nano- and ultrananocrystalline refers to the size of the crystals: microcrystalline diamonds are about 1 μm , the size of nanocrystalline diamonds is between 5 nm and 100 nm, and the ultrananocrystalline diamonds are smaller than 5 nm (Gibson *et al.*, 2009). Polycrystalline diamonds are usually diamond crystals grown on a certain nucleus, such as ultrananocrystalline diamond particle or an impurity grain. Nanodiamonds can be grown either as individual nanoparticles or as a film, usable as a coating of various surfaces.

Diamond nanoparticles (Figure 8) have the same crystal order and hybridization as bulk diamonds but they are, in addition, covered by a non-diamond carbon shell. This shell is usually composed of sp^2 hybridized carbon, usually various forms of graphite, nano-onions or amorphous carbon (Schrand *et al.*, 2009). It is difficult to determine the exact percentage of the surface which is covered by this shell. Some authors believe that the diamond core is covered only partially by this shell (Panich *et al.*, 2006; Schrand *et al.*, 2009) and consequently some bare plots are left which therefore can be hydrogenated or otherwise chemically modified (Panich *et al.*, 2006). However, well-purified nanodiamonds have almost perfect crystalline structure containing negligible fraction of non-diamond carbon. Nanodiamond cores inside a multi-walled fullerene structure, called bucky-diamonds, have been also prepared (Banhart and Ajayan, 1996).



A **B**

Figure 8 A) HRTEM image of a nanodiamond particle **B**) The SEM micrograph of NCD film deposited on SiO_2 matrix (Rezek *et al.*, 2010)

Nanodiamonds can be grown also in the form of a thin film attached to a matrix which can be SiO_2, stainless steel, amorphous carbon, graphite etc. (Jakubowski *et al.*, 2004; Rezek *et al.*, 2010; Petrak *et al.*, 2011). The structure of nanodiamond films is polycrystalline, as seen from Figure 8.

Apart from genuine nanodiamond films, there also exist so called diamond-like carbon films (DLC films). This material is amorphous, with significant fraction of sp^3 carbon bonds. The physical and mechanical properties of this material are similar to those of diamond (Grill, 1999; Lifshitz, 1999) and also the matrices used for their growth are identical (Ishihara *et al.*, 2006; Zhao *et al.*, 2007; Liu *et al.*, 2008; Zhou *et al.*, 2008; Marciano *et al.*, 2009a; Marciano *et al.*, 2009b; Marciano *et al.*, 2009c; Shao *et al.*, 2010; Marciano *et al.*, 2011).

In general, nanodiamonds have similar properties to those of bulk diamonds, such as high durability, hardness, chemical stability, high thermal conductivity and transparency. Nanodiamond and DLC films possess also these properties. In addition, they are "nanosmooth" i.e. they have very low friction coefficient (Grill, 1999; Lifshitz, 1999).

2.1.1. Stability

The size of nanoparticles is one of the criteria for their stability. From the bulk carbon materials, graphite is the most stable carbon allotrope. However, with decreasing size, nanodiamonds, bucky-diamonds and fullerenes (in the order given) became more stable than graphite (Barnard *et al.*, 2003). Concerning nanodiamonds, conversion of sp^3 carbon to sp^2 carbon together with attaching functional groups was reported to increase the stability of these nanoparticles (Mochalin *et al.*, 2012).

Temperature and pressure also affect the stability of carbon nanomaterials. For example, nanodiamond particles are often prepared by exposing fullerenes or CNTs to high temperature and high pressure (Ma *et al.*, 1994; Cao *et al.*, 2001) and DLC films change into graphite-like structures by thermal activation.

2.1.2. Functionalization and doping

Great advantage of carbon nanomaterials is their rich surface chemistry. The functionalization of carbon nanomaterials by various chemical groups modifies their properties and thus enhances the range of their possible applications.

The most commonly used functionalization is hydroxylation or carboxylation of the nanomaterial surface. This modification is often employed on purpose to enhance the solubility of nanomaterial in many biological studies (McHedlov-Petrossyan *et al.*, 1997; Deguchi *et al.*, 2001; Foley *et al.*, 2002; Tsao *et al.*, 2002; Sayes *et al.*, 2004; Lyon *et al.*, 2005; Lyon *et al.*, 2006; Tang *et al.*, 2007; Aoshima *et al.*, 2009; Arias and Yang, 2009; Akhavan and Ghaderi, 2010; Akhavan and

Ghaderi, 2012). However, it also can occur spontaneously, for example during purification process (Dolmatov, 2001).

Nanodiamond particles and DLC films can also be functionalized by oxygen containing groups using oxygen plasma treatment. The oxygenated material is rougher, superhydrophilic and more desorbed (Marciano *et al.*, 2011). NCD and DLC films can also be treated by hydrogen plasma which results into attachment of hydrogen atoms to the material surface. So modified films exhibit higher hydrophobicity and higher surface energy (Zhou *et al.*, 2008; Rezek *et al.*, 2010).

The attachment of NH_2- group, that confers positive charge to the nanoparticle, is also very frequently used modification (Tang *et al.*, 2007; Arias and Yang, 2009). Also other, very complex functional groups can be attached to the nanomaterial surface (Mashino *et al.*, 1999; Mashino *et al.*, 2003).

The nomenclature of modified nanomaterials is not unified: for example fullerene functionalized by OH- groups can be called hydroxyfullerene, fullerenol or fullerol (Kokubo *et al.*, 2008; Brunet *et al.*, 2009).

So called doping is used particularly in the case of nanodiamond and DLC films. The difference between functionalization and doping is that functionalization is related just to the surface of the nanomaterial while during doping some of the carbon atoms of the crystal structure are replaced by atoms of another element (e.g. silicon). Doped materials differ from the pristine materials in surface roughness, sp^2/sp^3 ratio (in the case of DLC films), or the values of surface energy (Ishihara *et al.*, 2006; Zhao *et al.*, 2007; Liu *et al.*, 2008; Zhou *et al.*, 2008; Shao *et al.*, 2010). The roughness of the material increases with higher content of Si or F content (Ishihara *et al.*, 2006; Zhao *et al.*, 2007). High content of Si also increased the sp^2/sp^3 ratio and lowered surface energy (Liu *et al.*, 2008; Shao *et al.*, 2010). Surface energy was lowered also by nitrogen doping (Liu *et al.*, 2008).

2.1.3. Absorption properties

The characteristic properties of nanoparticles, including these of carbon nanomaterials, are also great specific area and high absorption ratio. CNTs and fullerenes exhibit higher absorption ratio than activated carbon, which is used as a common absorbent (Li *et al.*, 2003; Gupta and Saleh, 2013). CNTs can absorb metal ions (e.g. copper, lead, nickel, chromium, arsenic, cadmium, zinc or cobalt), organic compounds (e.g. 1-naphthol, benzene, ethylbenzene, trichloroethylene, acetone, dioxin) or pesticides (e.g. carbofuran, iprobenfos, parathion-methyl, prometryn, fenitrothion, etc.)(Gupta and Saleh, 2013). Also antibiotics, such as sulfonamide, sulfamethoxazol or tetracycline, can be absorbed on the surface of CNTs (Ji *et al.*, 2009a; Ji *et al.*, 2009b; Gupta and Saleh, 2013). The absorption volume is higher for SWCNTs than for MWCNTs. The reason may be that molecules of antibiotics are quite big and thus cannot access the inner surfaces of MWCNT (Ji *et al.*, 2009a).

Absorption volume of fullerenes is lower than that of CNTs. Substances that can be absorbed on fullerene molecules are usually aliphatic, cyclic or aromatic compounds, which are also environmental contaminants, e.g. naphthalene or 1,2-dichlorobenzene (Gupta and Saleh, 2013).

Also nanodiamonds possess great specific area (Dolmatov, 2001) and could be used as absorbents. Among other compounds, they can absorb various biologically relevant molecules such as toxins, e.g. aflatoxin B1 or ocharotoxin A, or therapeutic proteins (Mogilnaya *et al.*, 2010; Gibson *et al.*, 2011).

2.1.4. Solubility

All pristine carbon nanomaterials mentioned in this thesis are insoluble in water and other polar media (Ruoff *et al.*, 1993; Ham *et al.*, 2005; Ozawa *et al.*, 2007) where they form aggregates of variable size in dependence on salt concentration (Deguchi *et al.*, 2001; Lyon *et al.*, 2005) and method of preparation (Neverovskaya *et al.*, 2004; Lyon *et al.*, 2006). Their low solubility is the reason why preparation of stable colloid solutions (chapter 2.1.4.2) is so important for their use in biological experiments.

2.1.4.1. ζ-potential

ζ-potential (zeta potential or electrokinetic potential) of aggregates is very important property of nanomaterials. A charged particle located in polar media is surrounded by a layer of solvent. This layer can consists of two parts: in the inner region, also called Stern layer, the molecules are strongly bound while in the outer, diffuse region the molecules are less firmly associated and less organized. In this outer region we can find a boundary (called hydrodynamic slip plane) between the molecules of solvent that form a stable entity with charged particle and molecules that moves independently around the particle. If the particle moves because of gravity or electric field, molecules of solvent located within this boundary stay associated with the charged particle. The potential at this boundary is called ζ-potential (Figure 9). The value of this potential can serve as a tool for prediction of stability of suspension in polar media: if the absolute value of ζ-potential is below 30 mV, the suspension is considered to be unstable. The value of ζ-potential can be influenced by many factors, such as pH, ionic strength and the surface chemistry of the particle.

Carbon nanomaterials originally have no charge, hence they should have zero ζ-potential and their suspensions are very unstable and therefore nanoparticles tend to aggregate promptly (Hu *et al.*, 2005; Kang *et al.*, 2007; Arias and Yang, 2009; Liu *et al.*, 2009; Yang *et al.*, 2010). However, it has been reported that carbon nanomaterials dispersed in water can be negatively charged. SWCNTs have a negative potential very close to zero in neutral pH (Hu *et al.*, 2005) and fullerene was found to have low ζ-potential about -9 mV (McHedlov-Petrossyan *et al.*, 1997) which is in accord with the low dispersibility of these molecules in water. Nevertheless, a later study reported different values, in

particular -30 mV for fullerenes (Deguchi *et al.*, 2001). Diamond nanoparticles also exhibit negative potential. It is believed that in the case of nanodiamonds this negative charge could result from the presence of hydroxyl group and other oxygen containing functional groups which are usually found on the surface of commercially supplied nanodiamond powder (Boehm, 2002; Williams *et al.*, 2010). In general, carboxyl, lactone, phenol and lactol groups contribute to the acidic character of the particle (Gibson *et al.*, 2009). On the other hand, the origin of basic character has not been clearly determined yet. It has been suggested that it could be caused by presence of diketone or quinone groups, pyronelike groups and electrostatic interaction of protons with the π-electron system of the graphene structures (Gibson *et al.*, 2009). An example of positively charged carbon nanoparticle could be nanodiamond particles treated with hydrogen. The ζ-potential of such nanoparticles is positive along almost whole pH scale (Williams *et al.*, 2010).

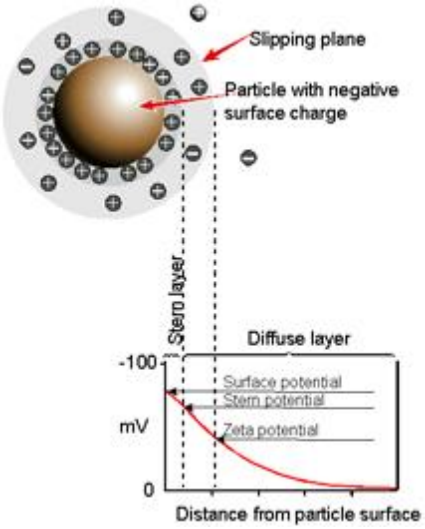


Figure 9 The potential at different distances from the charged particle http://www.nbtc.cornell.edu/facilities/downloads/Zeta%20potential%20-%20An%20introduction%20in%2030%20minutes.pdf

ζ-potential is influenced also by ionic strength. Nanoparticles with higher value of ζ-potential have no tendency to aggregate because they repel each other. However, the ions present in solvent can eliminate the interference from the charge on the particle surface and thus enable nanoparticles to aggregate. This is in agreement with the work of Fortner *et al.* (2005) who showed that negatively charged fullerene formed more aggregates in solutions containing higher concentrations of salts.

2.1.4.2. Preparation of stable colloids

In general, two possible techniques can be used to converse carbon nanomaterials to water solutions or at least to decrease the amount of aggregates formed. First is the chemical modification of the particle surface by attaching functional groups (chapter 2.1.2). The alternatives comprise sonication, stirring, employment of different chemicals such as THF or using detergents that cover the nanoparticles and prevent them from aggregation.

Using detergents is a good method how to prepare stable dispersions. There exist suitable detergents almost for every carbon nanomaterial. Stable dispersion of CNTs can be prepared by using sodium dodecylbenzene sulfonate (SDBS), sodium dodecyl sulfate (SDS), lithium dodecyl sulfate (LDS), tetradecyl trimethyl ammonium bromide (TTAB), sodium cholate (SC) (Sun *et al.*, 2008), 1-methyl-2-pyrolidone (NMP) (Ham *et al.*, 2005) or biocompatible Tween 20 (Liu *et al.*, 2009). For solubilization of fullerene, polyvinilpyrrolidon (PVP) is used which can be applied also in biological experiments (Lyon *et al.*, 2006). Nanodiamonds can be solubilized by using sodium bis(2-ethylhexyl) sulphosuccinate (AOT), Triton X-100, polyvinil alcohol (PVA), cetyltrimethylammonium bromide (CTAB), and tert-octylphenoxy poly(oxyethylene)ethanol (IGEPAL). However, the dispersions prepared this way are stable for approximately one week only (Maitra *et al.*, 2008).

Another method of colloid preparation, used especially in the case of fullerenes, is dissolving the nanomaterial in nonpolar media which is afterwards gradually replaced by water. In biological studies, tetrahydrofuran (THF) is very often used as nonpolar medium for this purpose. The diameter of aggregates obtained by this procedure is usually above 50 nm (Deguchi *et al.*, 2001; Fortner *et al.*, 2005; Lyon *et al.*, 2005; Lyon and Alvarez, 2008; Lyon *et al.*, 2008a; Lyon *et al.*, 2008b; Spohn *et al.*, 2009). In my opinion, the usage of this method is quite disputable: it results in nano-fullerene aggregates of the size which can be also obtained by using other methods, such as sonication or stirring (discussed in the next paragraph) which seems to be much more suitable for biological experiments because they simulate better the situation which could occur in the nature. Chemical compounds, such as THF, could theoretically intercalate into the fullerene aggregates and influence the results of the experiments on biological material. From this point of view, it seems that using other, chemical-compound-free methods is much better choice.

Sonication can be used for all carbon nanoparticles for disintegration of big aggregates (Deguchi *et al.*, 2001; Neverovskaya *et al.*, 2004; Lyon *et al.*, 2006; Foldvari and Bagonluri, 2008; Beg *et al.*, 2011; Yu *et al.*, 2012). Sonication of fullerenes results in formation of aggregates with uniform size about 50 nm (Lyon *et al.*, 2006). In the case of CNTs, the sonication time affects the distribution of nanotube lengths, while the sonication power applied affects both their diameter and length (Yu *et al.*, 2012). Also for dispersing nanodiamond particles the time of sonication is an important factor. The behaviour of nanodiamonds while sonicated, as described by Neverovskaya and co-workers, is rather peculiar: after first five minutes of sonication, the aggregates break to smaller ones but after additional 5 minutes (10 minutes in total) the smaller aggregates start to cluster again into bigger ones and the colloid becomes very unstable. After additional 10 minutes of sonication (20 minutes in total) the clusters disintegrate once more and this form of colloid is stable for next 2 hours after which spontaneous aggregation occurs again (Neverovskaya *et al.*, 2004). However, sonication itself cannot ensure the solubilization into the primary particles of nanodiamonds. Some authors suggest using zirconia beads for improving the result of high power sonication (Ozawa *et al.*, 2007).

The last method for preparing stable colloids or for breaking bigger aggregates to smaller ones is stirring in water. This method well simulates the situation that could occur in nature if carbon nanoparticles were released to the environment as pollutants and is also suitable for biological experiments. To my knowledge, stirring is not used for disintegrating the clusters of nanotubes but it has been applied with satisfactory results in the case of fullerenes and nanodiamonds. Fullerene aggregates prepared by this method have various diameters above 30 nm (Lyon *et al.*, 2006). For nanodiamonds, stirring was found to be more effective than sonication (Ozawa *et al.*, 2007). Unfortunately, this method is quite time-consuming – it usually takes days to prepare stable colloid solution (Cheng *et al.*, 2004).

While the product of covering the nanoparticles by detergent is stable colloid, stirring, sonication and employment of THF produce small aggregates.

3. The interaction of carbon nanomaterials with bacteria

The physical, chemical and structural properties of carbon nanomaterials have been intensively studied for many years and their properties are quite well characterized. However, their biocompatibility and especially their antibacterial potential have been much less explored so far.

Nanomaterials are of so small size that they are comparable to cellular structures. For example, fullerene C_{60} has diameter one order of magnitude smaller than is the thickness of the lipid bilayer and the diameter of SWCNTs is comparable to that of DNA double helix. Such dimensions predestine nanomaterials to interact with cells on molecular level.

Cytotoxicity (to eukaryotic cells) of carbon nanomaterials is explored better than their effects against bacteria. Nanodiamonds and graphene seem to be quite biocompatible, i.e. harmless to eukaryotic cells (Kalbacova *et al.*, 2010; Chang *et al.*, 2011; Mochalin *et al.*, 2012; Yang *et al.*, 2013) while fullerenes and CNTs are quite cytotoxic (Foley *et al.*, 2002; Sayes *et al.*, 2004; Sayes *et al.*, 2005; Lewinski *et al.*, 2008). It is still under discussion, whether nanodiamonds negatively affect bacteria, however it seems that graphite-derived nanomaterials have strong antibacterial properties. Five possible mechanisms are considered to be involved in antibacterial action of nanomaterials in general: the cell wall damage or total cell disruption caused by direct contact with the nanomaterial, production of reactive oxygen species (ROS), protein oxidation, impairment of metabolic pathways (especially those of energetic metabolism) and releasing toxic ions. The possible toxic action of carbon nanomaterials against bacteria involves all these mechanisms except of releasing hazardous constituents, which usually underlies the antibacterial properties of nanomaterials based on silver or heavy metals (Klaine *et al.*, 2008).

Antiadhesive properties of carbon nanomaterials, especially nanodiamonds and DLC films, are also of great interest. Bacterial adhesion to various surfaces is a fundamental problem not only in medicine but also in industrial tubing and water treatment devices, therefore effective antiadhesive and/or antibacterial coatings are highly needed.

3.1. The different susceptibility of gram-negative and gram-positive bacteria

The susceptibility of different bacteria to the negative effects of carbon nanomaterials depends on their cell wall morphology (gram-positive vs. gram-negative) and even on the bacterial species. It has been reported that gram-negative *Escherichia coli, Pseudomonas aeruginosa* and *Salmonella typhi* were more resistant to graphene, SWCNTs or carboxyfullerene compared to gram-positive *Bacillus subtilis, Staphylococcus aureus, Streptococcus pyogenes, Entherococcus fecalis* and *Streptococcus pneumoniae* (Tsao *et al.*, 2002; Tang *et al.*, 2007; Liu *et al.*, 2009; Akhavan and Ghaderi, 2010). Tsao

and co-workers (2002) also showed that minimal inhibition concentration (MIC) of carboxyfullerene $C_{63}(COOH)_6$ differs for different bacterial species and even different strains of one species (chapter 3.2.1). The authors tested many gram-positive and gram-negative bacterial species and determined the MIC 5-50 $mg.l^{-1}$ for gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Entherococcus fecalis* and *Streptococcus pneumoniae*). Gram-negative bacteria were not killed in their experiments even by the dose of 500 $mg.l^{-1}$. The results are summarized in Table 1. Pristine fullerene exhibited the same MIC for gram-negative *E. coli* and gram-positive *B. subtilis* (Lyon *et al.*, 2005).

Only little is known about interactions of bacteria with nanodiamonds. The recent study of Sawosz and co-workers (2011) showed that nanodiamond particles tend to attach to the outer cell structures of both gram-negative *Salmonella enteritidis* and gram-positive *Listeria monocytogenes* – see Figure 10. In the pilot study of our laboratory, nanodiamond particles have been shown to inhibit growth of *E. coli* on solid medium (Beranová *et al.*, 2012).

Bacteria (mg/L)	MIC (mg/L)	MBC
Gram-positive cocci		
Staphylococcus		
S. aureus 01	50	50
S. aureus 02	5	50
S. epidermidis 01	5	50
S. epidermidis 02	5	50
Group A streptococci		
S. pyogenes A-20	5	50
S. pyogenes SW507	5	50
S. pyogenes NZ-131	5	50
S. pyogenes SW510	5	50
Group B streptococci		
no. 543	50	50
no. 544	5	50
no. 567	5	50
Group D *Enterococcus*		
E. faecalis 002	50	50
E. faecalis 003	50	200
E. faecalis 004	5	50
E. faecalis 005	50	200
E. faecalis 474	5	5
E. faecalis 487	5	50
E. faecalis 496	5	50
S. pneumoniae		
serotype 14	50	50
serotype 6	50	50
Gram-negative bacteria		
E. coli	>500	>500
P. aeruginosa	>500	>500
S. typhi (S3-120)	>500	>500

Table 1 Minimal inhibition concentrations (MICs) and minimal bactericidal concentrations (MBCs) of carboxyfullerene against various bacterial species and strains. Reproduced from (Tsao *et al.*, 2002)

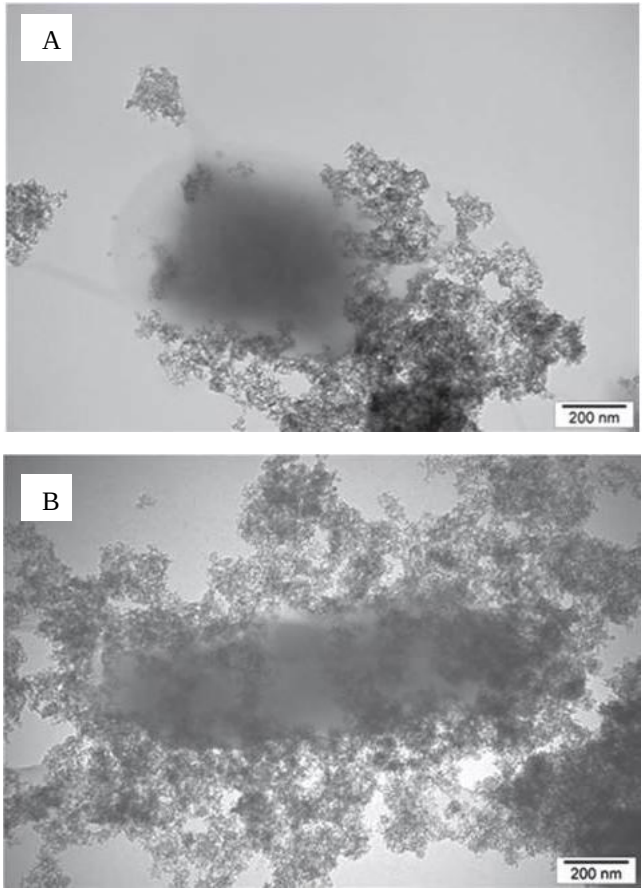


Figure 10 The TEM micrographs of nanodiamond particles attached to bacterial cells of (**A**) *Salmonella enteritidis* and (**B**) *Listeria monocytogenes* (Sawosz *et al.*, 2011)

3.2. Antibacterial properties of carbon nanomaterials

The exact mechanism of antibacterial action of certain nanomaterials is not fully described yet. However, there are many theories and hypothesis which are more or less probable. As far as carbon nanomaterials are particularly concerned, it seems that mainly the direct contact and possibly also production of ROS and protein oxidation are involved in their antibacterial activity. The available information on possible mode of action of carbon nanomaterials against bacteria is summarized in the next sections.

3.2.1. Damage caused by direct contact

The direct contact seems to be one of the crucial factors in killing mechanism of carbon nanomaterials. The bacterial growth is more inhibited by the direct contact with the carbon nanomaterial compared with the situation when the same nanomaterial is just present in the bulk cultivation medium. It has been reported that fullerene dispersed in medium by employment of PVP (chapter 2.1.4.2), which is supposed to cover the fullerene surface, does not exhibit as high toxicity to bacteria as the fullerene dispersed by different method which leave the surface of the molecules uncovered (Lyon *et al.*, 2006).

Also the lower antibacterial activity of NH_2-functionalized nanotubes reported by Arias and Yang (2009) could be due to long hydrocarbon chains ($-CH_3(CH_2)_{16}CH_2-NH_2$) used in their experiment for attaching NH_2- group to the surface of CNT. This arrangement could protect bacteria against SWCNT toxic action, because the nanotubes were not in the close direct contact with the cell wall (Arias and Yang, 2009). Nevertheless, the effect of fullerene modified in similar way is completely different, although the $-NH_2$ groups were also attached by a quite long chain ($-COO(CH_2)_3NH_2$) (Tang *et al.*, 2007). The only difference, if any, could be the fact, that fullerene was functionalized just on its one side, while the CNTs were functionalized on the whole surface (Arias and Yang, 2009).

According to the study of Akhavan and co-workers (2011), it seems that the plane areas of graphene do not exhibit any bactericidal effects. During cultivation, the cells of *E. coli* were (spontaneously) wrapped by the graphene-oxide nanosheets. The wrapped bacteria were not able to grow in the medium but were still alive. After removing the graphene sheets by sonication, the bacteria were able to consume glucose and proliferate again (Akhavan *et al.*, 2011). In contrast, the edges of graphene sheet can damage bacterial cells. For example, *S. aureus* and *E. coli* exposed to the sharp edges of graphene sheets died rapidly (Akhavan and Ghaderi, 2010). The authors suggest that the physical interaction between the bacterial membrane and the edge of graphene sheet was necessary for killing of the bacteria.

Similar effect was observed in case of nanotubes: short MWCNTs were more toxic to bacteria if they were dispersed and uncapped (Kang *et al.*, 2009; Liu *et al.*, 2009). Some authors suggest that this effect occurs due to higher percentage of the sharp edges in case of short nanotubes in comparison to the long ones (Kang *et al.*, 2008). However, it must be noted that the study of Kang (2008) was performed on short SWCNTs and long MWCNTs which differed in certain important properties, such as diameter - SWCNTs have smaller diameter than MWCNTs (Beg *et al.*, 2011), number of layers and length. MWCNTs have been found less harmful to the bacteria than SWCNTs (Arias and Yang, 2009). This observation is also in agreement with the work of Liu *et al.* (2011), who obtained similar results with oxidized graphene and graphite.

Diameter was also shown to affect the antibacterial properties of carbon nanotubes. The thinner nanotubes were reported to be more toxic to bacteria than the thicker ones (Kang *et al.*, 2008). In contrast to the work of Kang *et al.* (2008), other authors reported that the antimicrobial activity of SWCNTs increased with their increasing length – see Figure 11 (Yang *et al.*, 2010). However, the mechanism of action in this case was probably different: the longer nanotubes were able to attach the bacteria and form clusters with them, while the shorter SWCNT self-aggregated and therefore did not interact with bacterial surface (Yang *et al.*, 2010). Attaching of carbon nanotubes to the bacterial surface have been also reported by other authors – see Figure 12 (Liu *et al.*, 2010).

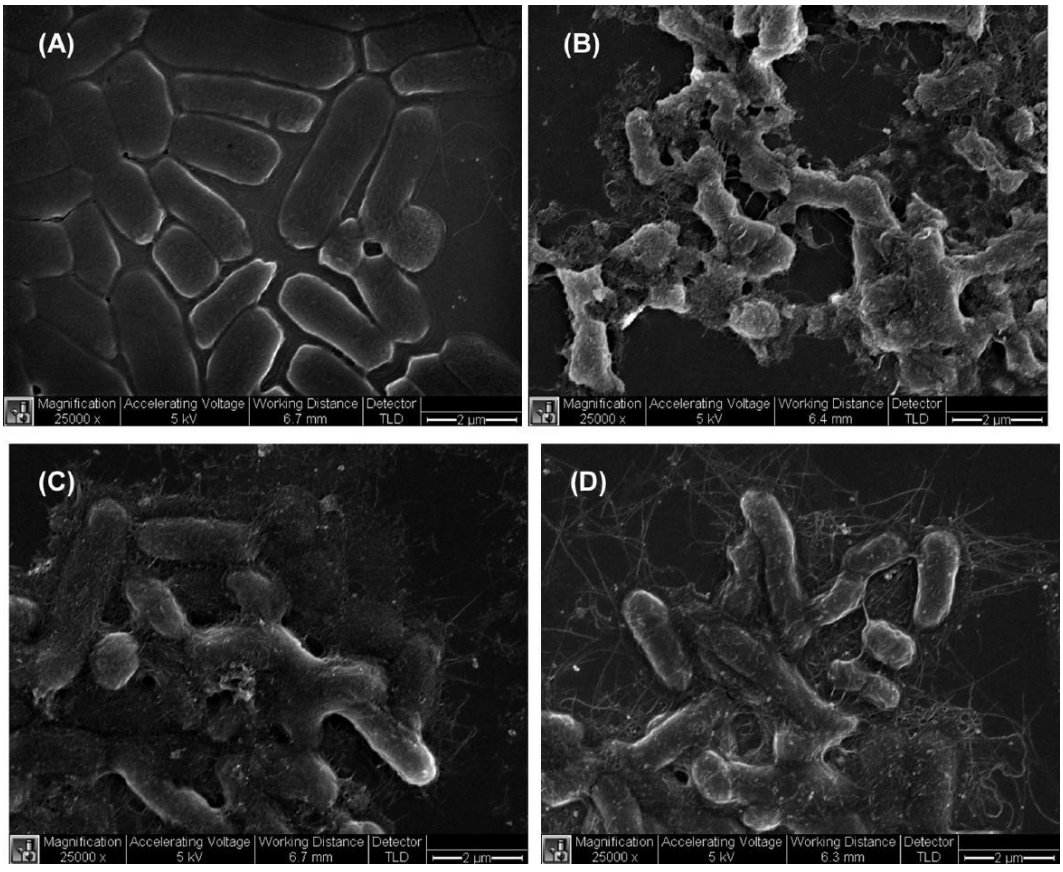
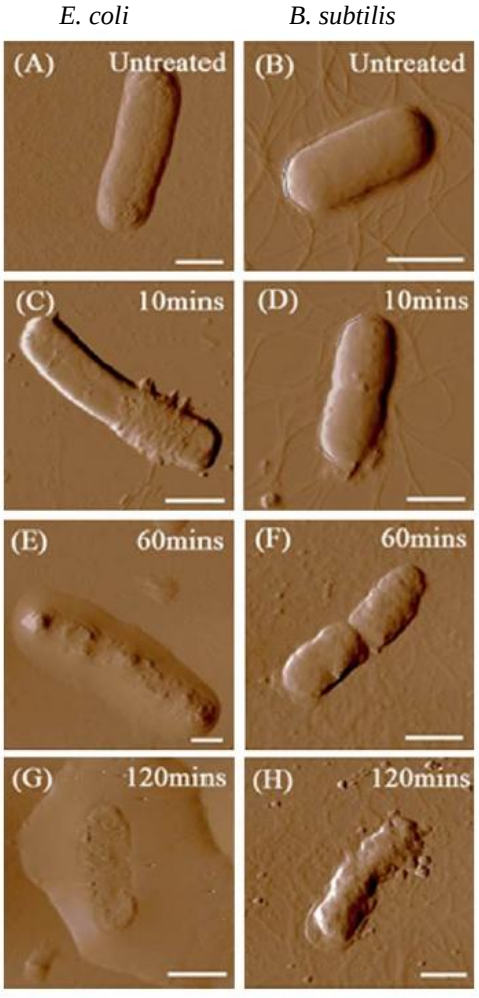


Figure 11 The representative SEM micrograph of *Salmonella* cells cultivated (**A**) without carbon nanotubes (CNTs) and with CNTs of various lengths: (**B**) <1 μm, (**C**) 1-5 μm and (**D**) ~5 μm (Yang *et al.*, 2010)

The mechanism by which direct contact confers the antibacterial activity of carbon nanotubes has not been confirmed and several hypotheses have been presented. The bacteria cultivated with carbon nanotubes very often lose their cellular integrity (Kang *et al.*, 2007; Kang *et al.*, 2008; Vecitis *et al.*, 2010) which is considered to be the result of morphological changes of the bacterial cell wall (Kang *et al.*, 2007; Kang *et al.*, 2008). Possible explanation of this observation is that sharper and smaller CNT could mechanically disrupt the cell wall. Liu and co-workers (2009) proposed that the nanotubes could behave as „nano-darts“ and kill bacteria by puncturing their cell wall. The authors discovered that increasing SWCNT concentration and higher shaking speed during incubation resulted in lower viability of bacterial cells. Even though their presumption corresponds with the experimental data, the physical puncturing of bacteria is not very probable mechanism of killing. For, in later study Liu *et al.* (2010) the same authors investigated bacteria by atomic force microscopy (AFM) and tried to puncture bacteria with super sharp silicon probe while measuring the force needed to puncture the cell wall and the number of punctures needed to kill one bacteria. They found out that to create a hole that would remain unchanged for at least 30 minutes, the force about 100 nN is necessary. Carbon nanotubes are not able to induce such force and therefore the cell wall is not probably damaged mechanically by them.



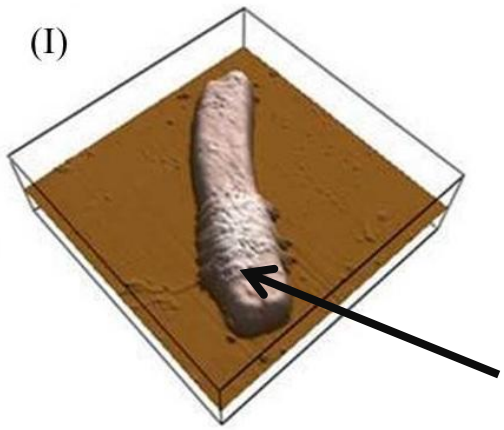


Figure 12 The ATM micrograph showing morphological changes of *E. coli* (**A), (C), (E)** and (**G**) and *B.subtilis* (**B**), (**D**), (**F**) and (**H**) cell ncubated with CNTs for different times.
(**I**) the 3D image of the *E. coli* cell of created on the base of the image (**C**). The authors (Liu *et al.*, 2008) suggest that the cell is partially covered by CNTs (indicated by an arrow)(Liu *et al.*, 2008)

The association of fullerene with bacteria has been also reported (Lyon *et al.*, 2005). The stronger association has been observed in the case of gram-negative bacteria which could occur due to the presence of the outer membrane in gram-negative bacteria that contains proteins which can interact with fullerene molecules. Fullerenes also induce membrane stress (Tsao *et al.*, 2002; Fang *et al.*, 2007; Lyon and Alvarez, 2008) which could result into the loss of cellular integrity and the leakage of cellular content (Tsao *et al.*, 2002). The possible mechanism of action could also involve direct contact of fullerene molecules or their aggregates with the cytoplasmic membrane. Many authors reported that fullerene is able to attach to the membrane (Foley *et al.*, 2002; Tsao *et al.*, 2002; Wong-Ekkabut *et al.*, 2008; Chang and Lee, 2010). However, several authors negate the hypothesis of membrane disruption caused by fullerenes (Lyon *et al.*, 2005; Lyon and Alvarez, 2008).

Several computational models have been created in order to simulate the interactions of carbon nanomaterials with biological membranes and to identify the exact location of the material within them. As the research on the interaction of carbon nanomaterials with bacteria is by no means completed, models describing the interaction of carbon nanomaterials with bacterial cell wall are not available yet. Either, no models have been made for the behaviour of nanodiamonds.

In general, graphene, carbon nanotubes and fullerenes tend to stay inside the cellular membrane (Qiao *et al.*, 2007; Bedrov *et al.*, 2008; Chang and Lee, 2010; Titov *et al.*, 2010; Jusufi *et al.*, 2011; Kraszewski *et al.*, 2012). Graphene usually intercalates parallel to the membrane surface plane without substantial affecting of the membrane – see Figure 13 (Titov *et al.*, 2010).

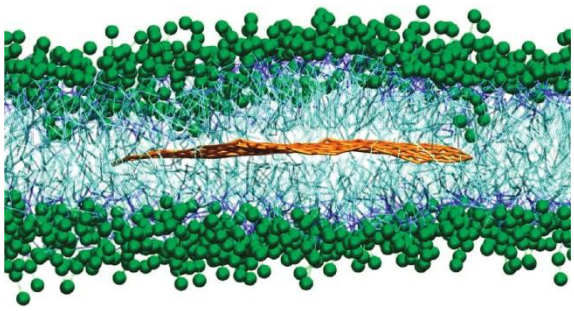


Figure 13 The position of a graphene sheet intercalated into the phospholipid bilayer (Titov *et al.*, 2010)

The penetration of short SWCNT can be divided into three steps: the floating on the membrane, the penetration itself - which is needle-like process - and sliding into the membrane core. The energy profile of this process is depicted in the Figure 14A. Two energetic minima are obvious: one is at the membrane surface (2.27 nm from the membrane centre) and second, more favourable, in the bilayer midplane. These two minima are separated by an energetic barrier which could be probably dependent on the tube diameter and length (Kraszewski *et al.*, 2012).

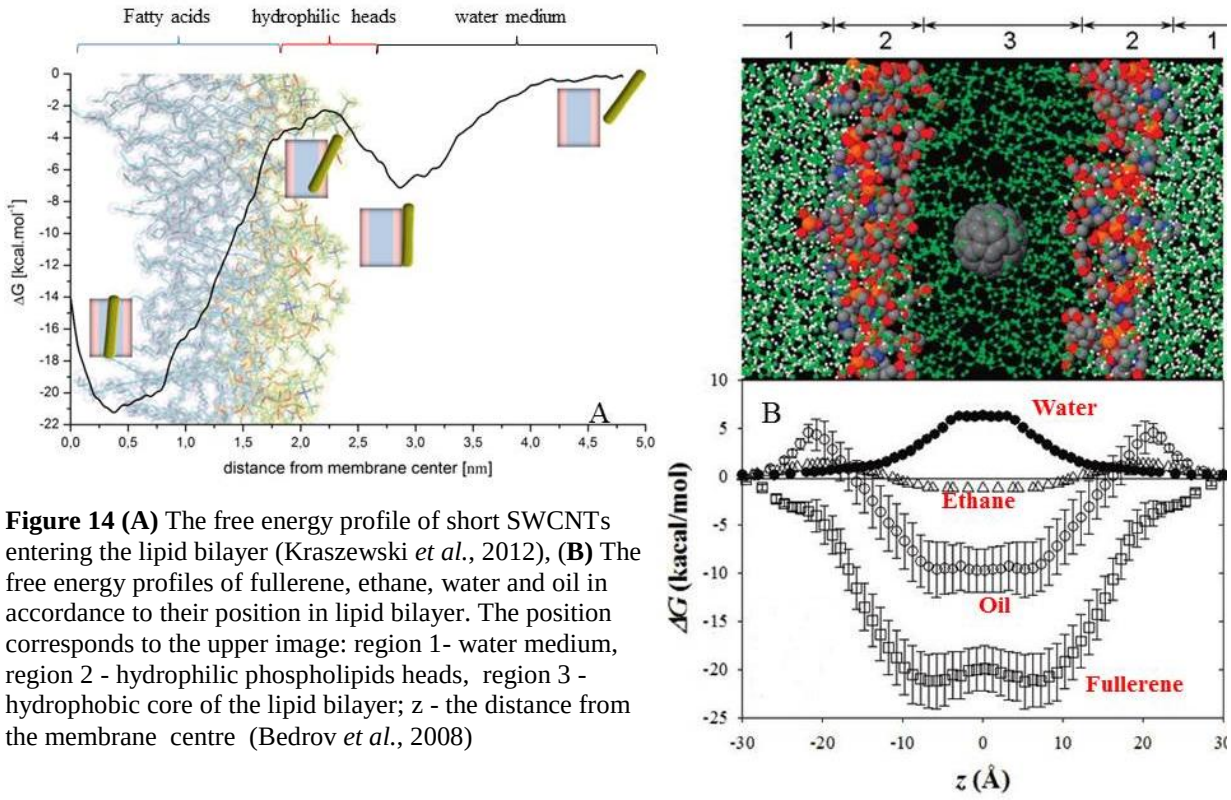


Figure 14 (A) The free energy profile of short SWCNTs entering the lipid bilayer (Kraszewski *et al.*, 2012), **(B)** The free energy profiles of fullerene, ethane, water and oil in accordance to their position in lipid bilayer. The position corresponds to the upper image: region 1- water medium, region 2 - hydrophilic phospholipids heads, region 3 - hydrophobic core of the lipid bilayer; z - the distance from the membrane centre (Bedrov *et al.*, 2008)

The energy profile of pristine fullerene C_{60} entering the membrane is very similar to that of the short SWCNT (Figure 14B) with the minimum corresponding to the localization in the hydrophobic region of the membrane (Bedrov *et al.*, 2008). The fullerenes have been suggested to reside primarily in the hydrophobic region of the membrane adjacent to the hydrophilic heads of the phospholipids (Bortolus *et al.*, 2011). Because fullerene could be considered a nanotube with zero length, this effect is in agreement with the previously mentioned presumption of (Kraszewski *et al.*, 2012).

Because of the tendency of fullerenes to aggregate in the polar media, computer simulation was performed that models the mechanism of interaction of fullerene aggregates (nano-fullerenes) with lipid membrane (Wong-Ekkabut *et al.*, 2008) - Figure 15. The first step of the process includes spontaneous forming of a small pore in the region of lipid head groups which is readily filled by one fullerene molecule from the cluster. Next, this molecule penetrates towards the lipid tail region and is followed by the rest of the molecules from the cluster. Finally, the fullerene cluster disintegrates into individual molecules which remain dispersed inside the hydrophobic region of the membrane. This dispersion has been reported as energetically more favourable than clustering inside the membrane (Wong-Ekkabut *et al.*, 2008; Chang and Lee, 2010). No fullerene molecules were calculated to leave the hydrophobic region of the lipid bilayer which is in good agreement with experimental data published by (Tsao *et al.*, 2002; Lyon and Alvarez, 2008).

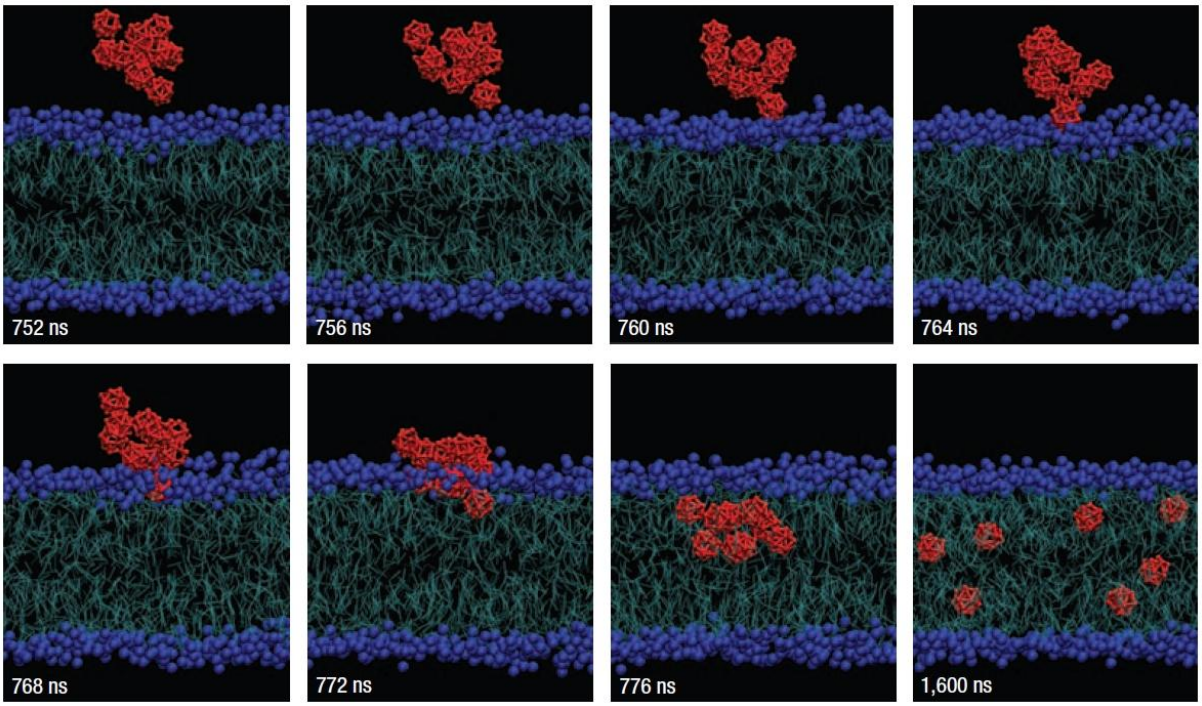


Figure 15 Penetration of fullerene aggregate into the lipid bilayer and its disintegration (Wong-Ekkabut *et al.*, 2008)

The behaviour of fullerene C_{60} in the membrane has also been investigated. The computational model of Qiao and co-workers (2007) showed that fullerenes inserted into the membrane can facilitate the formation of micropores which could contribute to the membrane leakage.

The dispersed fullerene molecules could influence membrane fluidity and eventually lead to membrane disruption. This hypothesis is in agreement with the work of Fang *et al.* (2007) who studied the composition of fatty acids in the membrane lipids of bacteria cultivated with different doses (0,01 - 0,75 $mg.l^{-1}$) of fullerene aggregates (nC_{60}). The authors have reported different behaviour of gram-positive and gram-negative bacteria. Gram-negative *Pseudomonas putida* responded to the low concentration of nC_{60} by increased production of saturated fatty acids and lower synthesis of unsaturated ones which altogether resulted in membrane rigidization. The higher doses induced the production of high melting cyclopropane fatty acid with the concomitant decrease of other fatty acid types, however the change of membrane fluidity was only minor. In case of *B. subtilis*, the low doses of nC_{60} invoked an increase of membrane fluidity, caused by the increase of anteiso-branched fatty acids in its membrane. When treated with higher dose of nC_{60}, *B. subtilis* responded by membrane fluidization via increased production of unsaturated fatty acids, however the level of both types of branched fatty acids dropped to extraordinary low level, almost to zero.

The effect of pristine fullerene has been also compared to that of fullerenol C_{60}-OH_{20} in a mathematical model of Qiao and co-workers (2007): while the C_{60} could contribute to the membrane leakage by formation of micropores, C_{60}-OH_{20} did not significantly diffuse to the membrane. This would explain the enhanced toxicity of carboxyfullerene $C_{63}(COOH)_6$ against gram-positive bacteria reported by Tsao *et al.* (2002) and discussed in the chapter 3.1. In their work, the cell walls of

gram-positive bacteria *S. pyogenes and S. aureus* incubated with carboxyfullerene had cottony surface (Figure 16), indicating the damage of the cell wall structure. In contrast, gram-negative bacteria possess the outer membrane which the water soluble derivative cannot penetrate and hence the bacterial cell wall was protected from destruction by carboxyfullerene.

In general, reduced or hydrogenated carbon nanomaterials, which are hydrophobic, are usually more toxic for bacteria than hydroxylated or carboxylated ones which exhibit hydrophilic properties. Lyon *et al*. (2005) showed that fullerene molecules hydroxylated by 22-24 OH- groups exhibited lower toxicity than aggregated fullerene. This is in agreement with the later work of (Tang *et al.*, 2007) who compared antibacterial action of C_{60}-OH, C_{60}-COOH and C_{60}-NH_2 against gram-negative bacteria *E. coli* and *Shewanella oneidensis*. He found that C_{60}-OH and C_{60}-COOH did not exhibit significant antibacterial activity while C_{60}-NH_2 inhibited bacterial growth even at concentrations about 10 $mg.l^{-1}$.

In contrast to the data discussed above, there exist also other works reporting contrary results. According to one study, the pristine C_{60} fullerene did not exhibit any antimicrobial activity while hydroxylated variants inhibited cell growth proportional to the surface hydroxylation of the fullerene molecule (Aoshima *et al.*, 2009).

As for the nanodiamonds, only scarce experimental data are available. Whether nanodiamonds can interact with the bacterial cell wall is not clear, but in the case of hydrophobic nanodiamonds it is possible that they may possess the ability to interact with the biological membranes. Also, it has been reported that nanodiamonds are able to interact with proteins and change their quaternary structure as has been shown on bovine serum albumin (BSA) (Wang *et al.*, 2011b).

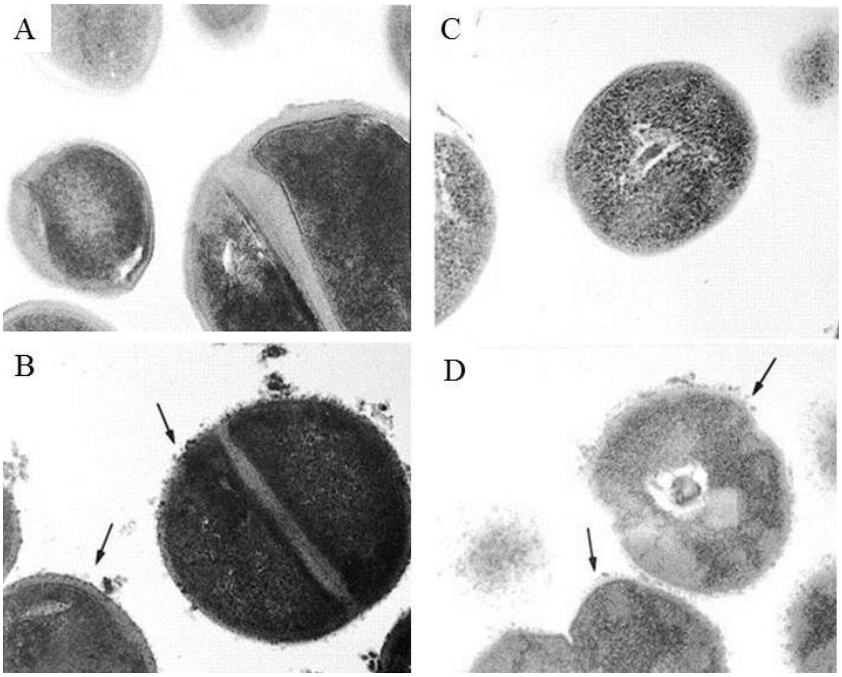


Figure 16 TEM images which illustrate the interaction of carboxyfullerene with the cell wall of gram-positive bacteria: (**A**) *S. aureus* untreated, (**B**) *S. aureus* treated with carboxyfulerene, (**C**) *S. pyogenes* untreated (**D**) *S. pyogenes* treated with carboxyfullerene. The arrows indicate the cell wall damage (Tsao *et al.*, 2002)

However, it is difficult to make any prediction on the exact character of interaction of nanodiamonds with bacterial cells because of the differences in the structure and properties between nanodiamonds and other - graphite-derived - nanomaterials. There is still lot of questions to answer and further research is needed.

3.2.2. The impact on the bacterial metabolism

The possible catalytic ability of nanomaterials and their impact on the metabolism have been also discussed. For example Akhavan and Ghaedri (2012) reported that bacteria of the genus *Shewanella*, which are capable of iron reduction (Fredrickson *et al.*, 2008), reduced graphene oxide originally nontoxic to bacteria, which was thereby converted to graphene that exhibits bactericidal effects. This reduction proceeds in anaerobic conditions only, probably because graphene is worse electron acceptor than oxygen (Wang *et al.*, 2011a). The authors suggest following mechanism of toxic action of graphene: the electrons may be transported from the inner space of bacterial cell by multihaem c-type cytochromes (periplasmic, MtrA, outer-membrane, MtrB and MtrC) which are intrinsic to all *Shewanella species* capable of metal reduction (Fredrickson *et al.*, 2008; Salas *et al.*, 2010) – Figure 17. The engagement of Mtr/Omc pathway in cytotoxic activity of graphene has been also confirmed by work of Wang *et al.* (Wang *et al.*, 2011a). It is also possible that very flexible graphene sheets attach to the cell, cover it and create a microenvironment with the reduced availability of oxygen (in which favours graphene as an electron acceptor and thereby enhances its bactericidal activity (Wang *et al.*, 2011a). In theory, similar mechanism could be involved in the cytotoxic action

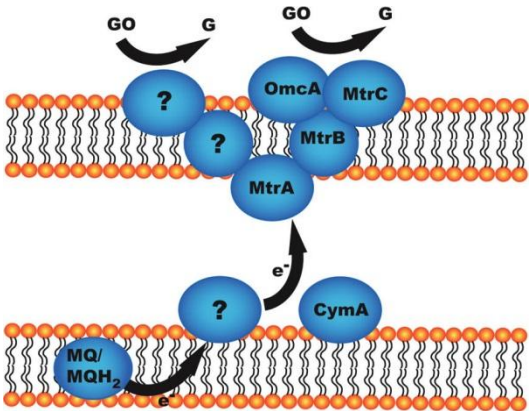


Figure 17 The Mtr/Omc pathway involved in graphene-oxide reduction in *Shewanella.* CymA cytochrome probably does not mediate electron flow from the quinone pool (MQ/MQH_2) to the periplasmatic cytochrome MtrA. G – graphene; GO – graphene-oxide; e^- - electron; ? – unknown carrier (Salas *et al.*, 2010)

of thicker CNTs because their surface has properties similar to those of graphene (chapter 2.1).

Another hypothesis on the mode of action of nanotubes against bacteria has been suggested by Vecitis *et al.* (2010) which have reported the high toxicity of conductive SWCNTs (against *E. coli*) in comparison with semiconductive SWCNTs, which did not show almost any toxicity. The authors

suggested that SWCNTs could penetrate the membrane and "short-circuit" the bacteria by acting as a conductive bridge over the insulating lipid bilayer and hence dissipating the cellular energy. In addition, the authors suggest that probable different level of toxicity of nanotubes, could be caused by different percentage of conductive and semiconductive carbon nanotubes in the samples used by different research groups. Similar mechanism of action is also suggested for graphene sheets as an alternative to the mode of action described above (Liu *et al.*, 2011).

Fullerenes have been also reported by certain authors to influence the metabolic activity of bacteria, while the others published contrary conclusions. Lyon and Alvarez (2008) have reported that nC_{60} reduced the membrane potential of gram-positive bacterium (*B. subtilis*) but not of the gram-negative *E. coli* and hypothesized that fullerene could uncouple the electron transport chain. As mentioned in chapter 3.2.1, fullerene is not probably able to cross the membrane hence the outer membrane of *E. coli* may serve as an additional protection against fullerene.

Also, the respiratory activity of *E. coli* and *B. subtilis* was reported to be inhibited by fullerene (Lyon *et al.*, 2005; Chae *et al.*, 2009). Likewise, certain more complex derivatives of carbon nanotubes could inhibit the respiratory chain (Mashino *et al.*, 2003). Flavin cofactor containing enzyme has been suggested as the inhibition target but this theory still remains to be confirmed.

3.2.3. Oxidative stress and production of reactive oxygen species

Another putative mechanism of toxic effect of carbon nanomaterials is via production of reactive oxygen species (ROS). The production of ROS could explain some of the confusing experimental data, such as toxicity of PVP coated fullerene (Brunet *et al.*, 2009).

The hypothesis that oxidative stress is involved in antibacterial behaviour of carbon nanomaterials is supported by the study of Kang *et al.* (2008) in which the authors focused on the changes of the *E. coli* gene expression in the presence of CNTs. The authors showed that genes of *soxRS* and *oxyR* system, which are related to the bacterial oxidative stress response, were upregulated when the cells were cultivated in the presence of both MWCNTs and SWCNTs. Two possible mechanisms have been suggested: the first involves the production of ROS, the second hypothesis assumes that carbon nanomaterials can act as oxidants. The production of ROS would support the hypothesis of some authors that direct contact of bacteria with nanomaterial is not implicitly needed for their antibacterial effect (Lyon *et al.*, 2006).

However, the ROS production is not probably involved in toxic action of graphene but has been widely discussed in the case of fullerenes. According to available literature, fullerenes and fullerenols are fotosensitive and capable of ROS production in the presence of light (Arbogast *et al.*, 1991; Kamat *et al.*, 2000; Pickering and Wiesner, 2005). This assumption is supported by the results of Sayes *et al.* (2004), who have reported the ability of nC_{60} to produce ROS in cell-free media in contrast to fully hydroxylated C_{60}. Nevertheless, the researchers from the Lyon's team showed

repeatedly that bacteria were killed by fullerenes even in the absence of O_2, which is - logically - the indispensable precursor for ROS production, and regardless to light (Lyon *et al.*, 2005; Lyon *et al.*, 2006). The exposure of bacterial cells to the ROS should also supposedly result in decrease of reductase activity (which is an indicator of normal electron transport chain function). However, no such decrease of reductase activity was observed after exposure of bacterial cells to nC_{60}. This would imply that ROS production is not involved in the case of antibacterial properties of fullerenes (Lyon and Alvarez, 2008).

Also another hypothesis that carbon nanomaterials may behave as oxidants is still under discussion. Many experimental studies suggest that carbon nanomaterials are able to oxidize proteins and peroxidize phospholipids (Kamat *et al.*, 2000; Sayes *et al.*, 2005), while other authors believe that carbon nanomaterials could behave as free radical scavengers (Wang *et al.*, 1999; Fenoglio *et al.*, 2006; Aoshima *et al.*, 2009).

It has been reported that putative ability to oxidize biomolecules is related to the conductive properties of particular carbon nanomaterial: for example, the oxidation of glutathion (GSH) was higher in the presence of conductive SWCNTs than in the presence of semiconducting ones (Vecitis *et al.*, 2010). The similar mechanism has been suggested for graphene sheets which exhibit excellent conductivity (Brownson and Banks, 2010; Yang *et al.*, 2013). The reduced graphene oxide sheets have been reported to be able of strong oxidation of GSH, which suggest that graphene could also be able to oxidize thiols and other cellular components such as lipids, proteins or DNA (Liu *et al.*, 2011). This result is in accordance with the previous work of the same authors, where they observed protein oxidation both outside and inside the bacterial cell in presence of sharp SWCNTs (Liu *et al.*, 2009). However, the authors themselves remark that the SWCNTs are just mild antioxidants.

The ability of fullerene to peroxidize lipids or oxidize proteins has been reported, too (Sayes *et al.*, 2004; Sayes *et al.*, 2005). These results are opposed by the work of Lyonand co-workers, who reported that fullerenes C_{60} does not cause either lipid oxidation (Lyon and Alvarez, 2008) or peroxidation, or oxidation of cytoplasmic proteins (Lyon *et al.*, 2008b). It should be noted that because fullerenes stay preferentially outside the cell (chapter 3.2.1), the oxidation of proteins would occur on the membrane interface (Lyon and Alvarez, 2008) and not in the cytoplasm.

However, according to results of some authors, SWCNTs could behave also as free radical scavengers as have been reported by Fenoglio and co-workers (2006). The reduction of free radicals resulted in acidification of solution, which could underlie the higher percentage of dead bacteria detected. This hypothesis is also supported by research of Arias and Yang (2009), who have observed the influence of the buffer type used on the bactericidal activity of CNTs. If the solution with SWCNT was not buffered, the pH decreased due to scavenging of •OH. Therefore, it is possible that the original cause of death of the bacteria during experiment was not ROS but too low pH.

The possible production of ROS reported by some authors for CNTs could also been attributed to the presence of substantial amounts of metal impurities in the CNT samples, which can vary between research groups.

3.3. Antiadhesive properties of carbon nanomaterials

Nanodiamond films are considered as promising materials for coatings of medical devices and articular implants. Therefore, the ability of NCD and DLC films to absorb proteins and protect the surfaces from the adhesion of microorganisms is intensively studied.

Indeed, surfaces coated with NCD or DLC films have been reported to inhibit bacterial adhesion – see Figure 18 (Jakubowski *et al.*, 2004; Ishihara *et al.*, 2006; Zhao *et al.*, 2007; Liu *et al.*, 2008; Zhou *et al.*, 2008; Marciano *et al.*, 2009a; Shao *et al.*, 2010; Marciano *et al.*, 2011; Medina *et al.*, 2012). However, many authors used materials doped by various elements such as silicon, fluorine or oxygen (Ishihara *et al.*, 2006; Marciano *et al.*, 2009a; Shao *et al.*, 2010) and different bacterial species for antibacterial properties testing (such as *E. coli, Salmonella sp., S. aureus, S. epidermidis, P. aeruginosa*), which makes the comparison of individual studies rather complex. In general, the extent of bacterial adhesion depends on the surface roughness, structural defects of the material sp^2/sp^3 ratio (in the case of DLC), hydrophobicity and surface energy.

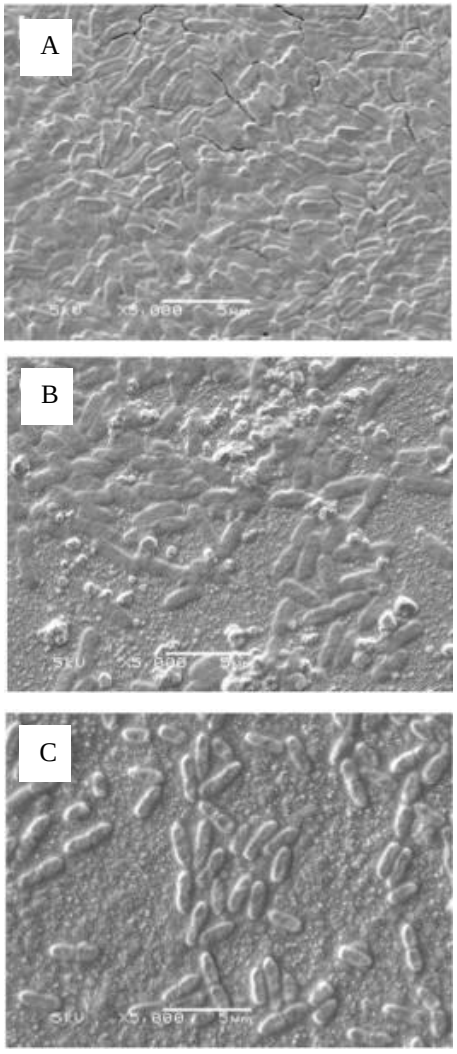


Figure 18 The SEM micrographs of *P. aeruginosa* cells adhered to the stainless steel (SS) surface **(A)** or SS coated with microcrystalline **(B)** or nanocrystalline **(C)** diamond film. Diamond coatings reduced the number of adhered bacteria (Medina *et al.*, 2012).

The surface roughness is influenced by the method of preparation and also by addition of other elements. DLC surfaces with higher content of Si or F are rougher than pristine DLC (Ishihara *et al.*, 2006; Zhao *et al.*, 2007). In both cases, the higher roughness prevented adhesion of bacteria (*E. coli, S. aureus, P.aerugiosa*) on the surface (Ishihara *et al.*, 2006; Zhao *et al.*, 2007; Liu *et al.*, 2008; Zhou *et al.*, 2008). Bacterial adhesion influenced by sp^2/sp^3 ratio has been studied especially on the Si-doped DLC films (Zhao *et al.*, 2007; Zhou *et al.*, 2008). Zhou *et al*. (2008) showed that adhesion of *E. coli* decreased with increasing sp^2/sp^3 ratio (which increased with higher Si content).

The ability of NCD and DLC films to inhibit bacterial

adhesion could be also attributed to their surface energy. Researchers have reported that the number of adhered bacterial cells (*E. coli, P. aeruginosa*) was indirectly proportional to the values of the surface energy (Liu *et al.*, 2008; Zhou *et al.*, 2008; Shao *et al.*, 2010). In contrast to these results, Marciano and co-workers (2011) reported that oxygen plasma treated DLC films that had rougher and superhydrophilic surface had the same antibacterial properties against *E. coli, P. aeruginosa, Salmonella sp.* and *S. aureus* in comparison to the untreated, relatively smooth and hydrophobic samples.

Many researches use the DLC or NCD films as carriers of other, usually toxic, elements or compounds, such as titanium oxide, silver or platinum. For example, TiO_2-doped DLC films exhibited strong antibacterial effect compared to untreated films (Marciano *et al.*, 2009c). Also doping DLC films by silver, which is known to be strong bactericidal agent, enhanced their antibacterial properties. However this effect seemed to be only short-termed, as after 24 hour incubation pristine DLC films exhibited higher antibacterial activity than Ag-doped films (Marciano *et al.*, 2009b).

4. Conclusions

The aim of this thesis was to investigate the mechanisms of toxic action of carbon nanomaterials against bacteria.

Due to their similar structure and properties, graphitic nanomaterials (CNTs, fullerenes, graphene) probably also share similar mechanism(s) of antibacterial activity. First hypothesis comprises the direct contact of the nanomaterial with bacterial cell, attaching and penetration of the outer bacterial structures. The nanomaterial can also reside in the bacterial membranes and influence their fluidity which can finally result in disruption of the cell. Conductive carbon nanomaterials can also cross the cell wall and cytoplasmic membrane and "short-circuit" the membrane potential which can lead to fatal energetic loss. Second hypothesis is based on the putative production of ROS, which could oxidize proteins and DNA.

In comparison to the graphite-derived nanomaterials, nanodiamonds are structurally different and therefore also their antibacterial properties (if any) probably differ from that of graphene, CNTs and fullerenes. However, the research on the antibacterial properties of nanodiamond particles, nanodiamond and DLC films is still at the preliminary stage and so there are not many information available in the literature. Those few studies published so far differ substantially in the experimental setup and also model microorganisms used. Doping of CNT and DLC films by various elements, which also is widely employed, offer many possibilities how to influence the final properties of the surface. These two factors rather complicate making any general conclusions from data published so far and intensive research is therefore needed to examine the suggested mechanisms thoroughly.

In summary, the studies published on the topic by different research groups often provide inconsistent and even contradictory conclusions. This inconsistency could be the consequence of two factors: (1) Incomplete specification of the carbon nanomaterial used: Nanomaterials purchased from companies that produce them commercially, which are often used in studies, can contain impurities which could substantially influence the results of experiments. Also disregarding of some important material characteristics (such as conductivity of carbon nanotubes) can lead to different interpretations of the results by different researchers. (2) The preparation method of stable colloid of carbon nanomaterials: Several different methods can be employed and the choice of method can significantly influence the experimental results. The disunity in sample preparation can therefore lead to differing results and problems with their reproduction by different authors.

Obviously, carbon nanomaterials – especially in respect to their effects on prokaryotic cells - still remains quite unexplored and provide a promising field for future research. The knowledge of the exact interaction mechanisms of these nanomaterials with bacteria could be later applied in medicine or biotechnologies and can help in discovery of new antibacterial coatings, drug delivery methods or virus inactivators.

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